A STUDY INTO THE RELATIONSHIP BETWEEN PHYSICAL ENVIRONMENT AND THE FUNCTIONALITY, STRUCTURE AND VIABILITY OF HEPATOCYTES IN-VITRO.

By

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BSc (Hons)

This thesis is submitted in partial fulfilment of the requirement for the degree of Master of Science in Biomedical Engineering

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August 2014

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed: _____ **Dated:** _____

Acknowledgements

Thank you to Professor Helen Grant, for her constant support and encouragement and for taking the time to guide me and reminding me to breathe.

Thanks must also go to Catherine Henderson for her advice, reassurance and guidance. Without her kind patience and tutelage I could not have finished any of this.

Thanks to Peter Agbekoh for his guidance with the HepG2 cells, his knowledge and experience was invaluable in handling the cells and collagen.

A special thank you to Selene Pellizzoni for her expertise and help.

Thank you to the whole lab for being so friendly and welcoming and always having the time to help.

I would also like to thank my family for always being understanding and willing to listen.

Abstract

Liver failure is a life threatening condition. A large problem in the manufacturing of synthetic liver tissue is maintaining the viability and differentiation state of the hepatocytes in-vitro. Cells are profoundly influenced by the stiffness of their ECM and deviations in in-vitro models from the in-situ environment can impact on their functionality.

The aim of this project was to investigate the cellular structure and shape, the actin cytoskeleton, the metabolic activity and viability of cells cultured in a collagen sandwich bioreactor and observe the differences compared with monolayer cultures.
HepG2 cells were chosen to model hepatocytes in comparisons between collagen sandwich bioreactors and monolayer conformations. This was done by sandwiching HepG2 cells between layers of collagen gels and visualising microscopically 24 hours after seeding them in monolayer. To visualise distribution and aggregation of cells they were treated with MTT, colouring viable cells blue. To determine overall cell structure the cell cultures were stained with Phalloidin-FITC (binds to actin), Calcein-AM and CFDA (a live dead stain) and fluorescent microscopy was performed.

The results presented show that human HepG2 cell morphology and intercellular communication is strongly influenced by both the environment and cellular densities of the culture. The findings in this research suggest that lower concentrations (0.5, 1 and 5 $x10^4$ cells/cm^2) of HepG2 cells seeded in collagen sandwiches are able to maintain a rounded in-situ morphology while culturing cells at a high density 10 $x10^4$ cells/cm^2 in a 3D scaffold allows for increased intercellular interactions and aggregation. This in turn may improve retention of in-vivo-like functionality and viability when culturing in-vitro. The increased cell to cell interaction and maintenance of in-vivo morphology could possibly help retain liver function in-vitro. This could be beneficial for future research aimed at improving the impact and efficiency of studies modelling detoxification of candidate pharmaceuticals and development of synthetic livers tissues.

Contents

1.0 Introduction

1.1 Liver

Liver is a very versatile organ which serves multiple functions in the human body. Research utilising liver cells, predominantly hepatocytes, has aided in drug development, tissue engineering and personalised medicine. The liver is essential for vital functions in mammalian organisms such as; digestion, detoxification, metabolism and the production of hormones. The most important functions of the liver are detoxification and metabolism, as the main site of drug metabolism (Gillette (1971)) and nutrient processing; liver is an imperative organ and therefore an indispensable model for pharmaceutical and medical research. Failure of the liver to carry out these functions results in severe illness and ultimately death of the organism. Liver failure occurs when the organ is damaged beyond repair and becomes unable to properly function. There are many causes of liver failure (Bernal and Wendon (2013)), all of which are life threatening and result in millions of deaths per year. Often the only possible treatment is a liver transplant operation (Palakkan et al (2013)). Therefore there is an increasing demand for donor livers and other tissue engineering alternatives. The low availability of donor livers and issues with post-operative host immune response causing tissue rejection have instigated research into synthetically reproducing liver function and tissues for implantation. Due to the integral functions of livers in living organisms a lot of research and development has been conducted to treat any ailment or decay that might interrupt or halt proper liver activity.

1.2 Liver cells

The liver is the largest internal organ of the human body, located in the abdominal cavity, it is connected to two blood vessels; the hepatic artery and portal vein which carry blood to and from the liver from multiple organs throughout the body. Within the liver the blood vessels form capillaries which specialise into lobular arrangements; these are the functional units of the liver. Lobules are mainly made of aggregations of parenchymal hepatocyte cells, but can also consist of small amounts of non-parenchymal; Kupffer and hepatic stellate cells.

Kupffer cells (KC), are macrophages found in liver sinusoids, adhered to the blood vessel walls which form part of the reticuloendothelial system. They function in the break down

and redistribution of blood components as well as acting as the first macrophage cell type to encounter bacteria, endotoxins or microbial debris from gastrointestinal or splenic systems (Bilzer et al (2006)). Kupffer cells make up 80-90% of the tissue macrophage cells in the body and are found in varying sizes throughout the liver, larger KC are found at the periportal region of the liver; they have high lysosomal enzyme activity and act as the first liver cells in contact with potentially pathogenic rich blood. Macrophage KC cells react to toxic agents via inflammatory mediators, growth factors, and reactive oxygen species (Roberts et al (2007)) to purify blood and maintain normal physiology and homeostasis of the liver.

Hepatic Stellate cells (HS), making up 5-8% cells of the liver and function by the regulated storage and release of retinoids (Geerts (2001)). Predominantly these cells remain in a state of quiescence but upon the recognition of liver damage signals secreted by hepatocytes (Yin et al (2013)) they activate and begin proliferating and performing chemotaxis. These cells are found around the sinusoid vessels and are believed to regulate blood flow, through their control of extracellular membrane (ECM) turnover, inhibition and secretion (Friedman et al (2003)). These HS cells have been shown to regulate liver stiffening, by coordinating collagen matrixes as a response to liver damage.

Hepatocytes are the most abundant and active cell type in the liver, making up more than 60% of the cell density and retaining a stem-cell like proliferation rate (Espejel et al (2010), Cheng and Gadue (2010)). Hepatocytes are smooth cubical cells of approximately 15µm when found in-situ, where they grow in monolayer plates sandwiched between sinusoid channels. Hepatocyte cells are the most metabolically active of the liver cells, functioning in many capacities; producing proteins such as serum albumin or fibrinogen, storing fatty acid carbohydrates and inactivating exogenous compounds found in the blood. As a synthesising cell, hepatocytes are vital to the whole body in their ability to produce a wide range of proteins, this is possibly due to large number of mitochondria, Golgi apparatus and endoplasmic reticulum (ER) that they possess. Hepatocytes are the main site of glucose storage and energy storage; converting carbohydrates to fatty acids while also being the central site of glucogenogenesis; the formation of carbohydrates from alanine, glycerol and oxaloacetate. Although the previous functions listed are important to continued healthy physiological function of the organism, the most researched and developed field studied has been hepatocyte activity in detoxification and immune response of xenobiotic exogenous compounds. Xenobiotic compounds are often dangerous or actively harmful to

the host system and therefore hepatocytes function to neutralize and remove these foreign compounds. There are two main mechanisms by which neutralization and expulsion can occur (Williams (1971)); the exogenous molecule is first ingested by the hepatocyte cell in which it will interact with any number of enzymes; these enzymes cause an oxidative, reductive or hydrolytic change to the compound adding a functional group, which is then modified to alter the polarity of the xenobiotic compound. This process increases the weight of the foreign compound and often makes the substrate more water soluble (Parkinson (1996)), allowing it to be inactivate to the cells and excreted through the bile channels. Once hepatocytes have altered or metabolised xenobiotic reagents they are able to package and remove the remnants from the body, this is carried out using the many smooth ER and Golgi apparatus that these cells possess.

1.3 Artificial Livers

Developing research into the treatment and modelling of liver related illnesses and functions has many advantages, but limitations caused by the availability and ethical concerns of fresh viable human liver as well as other viability issues of isolated primary hepatocyte cells in culture have caused problems. Experimentation on live subjects, while giving the advantage of often being a reliable mammalian in-vivo cell model of reaction to drugs and other stimulus, is ethically complicated and expensive. These studies are often limited in their scope and repeatability due to the low number of test subjects, legal regulations and ethical concerns. To accommodate for these disadvantages in-vitro cell models have been developed, utilising cells in controlled environments. These culturing techniques do not need as much maintenance as animal models, are not as expensive to run and can be repeated in near identical environments multiple times. By isolating and examining cultures of hepatocytes research has been able to test drug compatibility (Khetani and Bhatia (2008)), detoxification mechanisms (Behnia et al (2000)) and liver carcinogenesis (Barnett et al (2002)).

In-vitro analysis does not always necessarily model the true environment or function of liver cells in-vivo, there are multiple differences such as; ECM support and interactions, intercellular protein signalling or nutrient supply concentration and rate that are not reproduced as they are found in the host organism. These variations from in-situ environments can cause alterations in cellular morphology, function, behaviour and

viability which are detrimental for tissue engineering and drug development research. A major goal in tissue engineering is the development of transplantable synthetic whole livers and liver tissues; able to treat or replace failing liver cells, but to accomplish this multiple issues with in-vitro hepatocyte cultures need to be addressed. For example a large problem in liver tissue engineering is the loss of viability and dedifferentiation of primary hepatocytes isolated from livers and cultured in-vitro (Bhandari et al (2001)), this could be due to structural differences in the environmental composition surrounding cells as compared to those found in-vivo.

1.4 Extracellular Membrane

Cells are regulated and defined by their environment; the extracellular membrane is an amalgamation of compounds and chemicals which mechanically and chemically interact with the cells around them. ECMs are generally made of structural proteins; such as collagen and elastin, specialised proteins and proteoglycans; which act as signalling molecules on the ECM and on the cells. Studies have shown that removing the ECM from cells can cause loss of function, changes in morphology and cell death (Bhandari et al (2001)). In-vitro studies have shown that the introduction of ECMs to cells in culture can induce cell polarisation and cellular organisation in cells without ECM (Dunn et al (1989)) and cause retention of in-vivo phenotype of primary isolated cells. The components that make up cellular ECMs vary depending on the surrounding cell types in question and where they are located; the differences between ECMs can influence cell differentiation, function and viability. Hepatocytes are found within a unique extracellular membrane, whereas most epithelial cells are separated from endothelial cells by basement layers (BM), hepatocytes are separated by the space of Disse (Martinez-Hernandez and Amenta (1993)). The hepatocyte ECMs are interspersed with gaps and mainly consists of fibronectin and collagen (type I).

Cellular function has been shown to be directly influenced by intercellular environment, this regulation is due to chemical signals and mechanical stimuli caused by the composition and rigidity of the ECM (Khetani and Bhatia (2008)). The biochemical components, signalling molecules on the surface of the ECM, can influence multiple activities and states of cells such as moderation of cell-cell activity as well as osmoregulation. The rigidity of ECM

mediates mechanical forces acting upon cells while also regulating activation of specific receptors and intracellular signalling pathways. Signals by extracellular mechanical stimulus can cause multiple changes in; proliferation, differentiation, migration and aggregation states of cells (Mason et al (2013)). Primary hepatocyte cells isolated from tissue have been shown to lose function and morphology ~24 hours after being removed from their native environment, provided to them by their ECM (Grant et al (1985)). These two forms of environmental control, biochemical and mechanical, contribute to the regulation of differentiation, viability and function of all cells and without them hepatocyte cells will rapidly dedifferentiate and die.

1.5 In-vitro Models of liver

The majority of investigations utilising hepatocytes has been aimed towards toxicity and drug metabolism. There have been many in-vitro modelling systems designed to aid these studies and the most common are; isolated perfused liver, slices of liver, cell cultures and recently the development of more advanced artificial bioreactors or bio-artificial livers (BAL) all aiming to synthesize in-vivo ECM environments.

Isolated Perfused Liver

The isolated perfused liver (IPL) model allows for in-vitro assessment of whole liver function as well as flow rates in a controlled environment. This model retains the anatomical form, as well as ECM and 3D structure of in-vivo liver allowing for investigation of bile flow, hemodynamics and xenobiotic reaction mechanisms. Due to the maintenance of the ECM and intercellular interactions all in-vivo enzymatic activity is retained and can be examined which is useful in drug development (Bessems et al (2005), Groneberg et al (2002)). The most commonly used model of this kind is the isolated perfused rat liver, this is due to the problems ethically and logistically of acquiring enough human livers to be able to obtain reliable results (Bessems et al (2005)). Rat and human livers differ significantly in size, function and 3D architecture. Better analogous species are porcine, canine or bovine (Groneberg et al (2002))but these are not used due to the high cost and ethical issues they entail. In all IPL models the organs are maintained for a short time and it has been observed that functional integrity is never retained for a prolonged time (Groneberg et al (2002)). The research capacity of the IPL model is limited considering that livers being treated with a number of samples will become damaged and alter very significantly from in-vivo organs,

these samples then cannot be re-used. This model is useful for studies in multi-cellular functions as the cells are retained within in-situ conditions, but it is difficult to maintain and hepatocytes become non-functional or unviable rapidly. The short lifespan and inaccessibility of an IPL models mean that it is best suited for use in small studies investigating three dimensional architecture of liver or whole organ research monitoring fast acting chemicals.

Liver Slices

Slices of liver tissue are used as a model of in-situ liver, the premise of using this model are that cells retain their in-vivo environment as well as intercellular interactions as they did in IPL models but hepatocytes can be maintained and analysed in a more direct method. Liver slices are more accessible than full livers, coming from all species including humans, and multiple samples can be taken from one liver. The increased sample numbers allows for multiple compounds of different concentrations to be investigated and increased repeatability and therefore reliability of experiments ensues. This model retains lobule formations of hepatocytes but not the 3D anatomic structure of the liver and cannot be used in any flow experiments. Liver slices are able to retain in-vivo cytoarchitecture but dedifferentiate or become unviable after several days, which is an improvement of IPL which last for a couple hours (Groneberg et al (2002)), limiting their use as models (Khetani and Bhatia (2008)).

Cell Cultures

The most commonly used liver model is isolated cell cultures; these can be obtained from human sources and are therefore useful for drug development and toxicity experiments. Cells are usually isolated directly from tissue and then immediately cultured, cell samples can also be cryogenically frozen allowing for transport and storage. Using this model cellular defence and detoxification mechanisms can be identified and studied at a cellular level more directly and isolated cell mechanisms can be investigated by lowering the cell density of the culture. Research has shown that cell monolayers plated at higher densities retain in-vivo like polarity and high liver specific functions, possibly due to increased intercellular interactions (Moghe et al. (1997)). Due to the availability of the cells it is possible to research multiple chemicals at different concentrations, allowing for a broader scope of investigation.

This model does have its disadvantages as it is does not retain the 3D structure or ECM as found in-vivo and is unable to simulate the oxygen and nutrient transport perfusions found in the liver, causing cultured hepatocytes to becomes unviable rapidly. Cells are most commonly cultured on a polystyrene dish which may contribute to alterations by increasing stiffness of the ECM, thus inducing fibrosis, stressing of the hepatocytes and impacting on the functionality of the model. This isolated cell methodology of culturing hepatocyte cells has been proven to cause dedifferentiation and eventual unviability in cell cultures (Guillouzo (1998), Hewitt, N.J. et al (2007)), highlighting the need for development of further liver cell culture techniques.

3D Bioreactors

Current research utilises a method of seeding cells on a collagen gel then overlaying with another layer of collagen gel. This sandwiching of the cells between gels creates a closed system that allows for improved control of nutrient and oxygen flow, as well as facilitating cellular adhesion which helps maintain cell morphology, viability and function (Nahmias et al. (2007)). This model can be defined as a bioreactor as it conforms to Martin et al (2004) definition "as devices in which biological and/or biochemical processes develop under closely monitored and tightly controlled environmental and operating conditions (e.g., pH, temperature, pressure, nutrient supply, and waste removal)" (Martin and Vermette (2005)). Research utilising this sandwich bioreactor has shown that when mouse primary hepatocytes are sandwiched between collagen systems some degree of cell morphology and functionality remained including albumin secretion(Dunn et al (1989), Godoy et al (2009)), lipid and alcohol metabolism (Kim et al (2010)) and enzyme production and activity (Mathijs et al (2009)). There has been much work using mice as model organisms in describing the conformity and functionality of primary hepatocytes, showing that collagen sandwiching techniques produce more viable and functional tissue engineered products. Although sandwich bioreactors are superior for many purposes, at higher cell densities research has come across problems with mass transport of nutrients and oxygen to the cells occuring due to the barrier made by the top layer (Tan et al(2013)). This inhibition of oxygen and nutrient flow can cause cell stress and eventual dedifferentiation and unviability. Tan et al (2013) have shown that by sandwiching the hepatocytes between a synthetic extracellular membrane, containing proteins designed to facilitate nutrient flow and oxygenation, cell viability and function can be maintained for around 9 days. A

prominent example of this bioreactor has been developed to combat problems with the application of the model; including low cell numbers, low oxygen permeability of the collagen and issues with nutrient perfusion, issues which have dampened the impact in treatments such as BAL assembly. This recent study, Xia et al (2012), stacked 12 sandwiched layers of hepatocytes, allowing for the housing of 100 million (10^8) cells. They developed a nutrient flow method to allow optimal nutrient and oxygen content to perfuse over each sandwich layer while minimizing mechanical stress, thus increasing cell-fluid interactions, retained cellular polarity and function. This multi-sandwich bioreactor has been shown to maintain hepatocyte viability and enzymatic activity for up to 7 days. The advancement of this bioreactor may provide improved BAL treatment in replacing inactive liver functions for patients of liver failure. The sandwich model system allows for multidimensional investigation as flow-through between the collagen layers can be controlled, (Xia et al (2012)) and nutrient exposure through the individual permeable layers can also be regulated (Tan et al (2013)). This bioreactor model allows for maintained ECM, morphology, viability and function in a closed system.

By simulating the homologous environment within the liver, studies are able to investigate various aspects of cellular function, morphology and adhesion. There are multiple models each designed to simulate specific aspects of whole liver physiology, liver function or isolated cellular activity. Most models do not sustain cell viability and differentiation for very long, except the sandwich model that allows for prolonged retention of cell specialisation and viability. The sandwich bioreactor model also allows for a more accurate seeding density and containment in a 3D extracellular membrane. Due to the thin translucent permeable collagen top layer, cells can be stained and visualised by microscopy. To investigate the effects of variable seeding densities on cell characteristics; such as intercellular interactions, morphology and viability a 3D sandwich bioreactor model was chosen for the present study as it allows for controlled cell seeding density in each plate, repeatability and direct visualisation of results while retaining as much in-vivo environment as possible, provided by the 3D matrix provided by the two collagen layers.

1.6 Cell Sources for Hepatocyte Supply

The gold standard cell type for cellular liver function or synthetic liver development are human primary hepatocyte cells, cultured immediately after isolation. This potentially gives the most in-vivo like human liver cell behaviour. As discussed previously the most homogenous in-vitro models of liver architecture and function; IPL, liver slices and isolated liver cells all lose viability within a short time frame limiting the scope and duration of any research. Primary hepatocyte cells have been observed to dedifferentiate and lose liver enzymatic characteristics post seeding on collagen gels; therefore other cell types that are more resilient to environmental changes may provide a more robust model. To optimize experimentation utilising the sandwich bioreactor model, cell selection should allow for an accessible cell that while retaining morphological and functional identity in-vitro would facilitate for a vast range of research.

The HepG2 human hepatoma cell line, is a widely used hepatocarcinoma line that maintains some endogenous liver bioactivity in-vitro, and it has been shown to retain morphologic and enzymatic characteristics of primary parenchymal hepatocytes (Valentin-Severin (2003), Knowles (1980)). This line of immortalised cells were derived from the liver tissue of a human subject with a well differentiated hepatic-cellular carcinoma in 1979, and the cells are epithelial in morphology and have a chromosome number 55 (Constantini et al (2013)). Due to differences in hepatic metabolism and morphology between rat and human hepatocytes it is best to utilise human cells to ascertain the optimal validity in results (Chenery et al (1987), Richard et al (1991)). HepG2 cells, unlike other cancer cell lines, are differentiated to a degree, and have been shown to express some of the genotypical characteristics of primary hepatocyte cells (Gerets et al (2012), Sassa et al (1987)). These hepatoma cells do not form tumours in nude mice or express hepatotropic viral agents meaning that they are a benign strain to use as a model for hepatocytes (Javitt (1990)). HepG2 are widely used as models for primary liver cells due to their similar morphology and behaviour. They have been used to research enzyme activity, detoxification, DNA damage (O'Brien et al (2000)) and drug development as a replacement for unavailable primary hepatocytes. Some research has suggested over expression of liver function (Knetani and Bhatia(2008), Wilkening et al (2003)) while other papers have reported lower enzymatic activity(e.g. CYP3A4, CYP2A6, CYP2C9, and CYP2C19 (Westerlink and Schoonen (2007))), this shows slight variations of activity levels from wild-type which could possibly be due to

the tumour origin of the cell line. Although these variations in function may affect specific studies, by taking into consideration these variations, this should not impact on HepG2 as a comparative model for in-vivo hepatocyte cells, especially in studies monitoring morphology. Human HepG2 cells are simple to culture and can be maintained for a prolonged time (Gerets et al (2012)), they are widely accessible in large cell numbers and analogous to hepatocytes making this hepatoma cell line one of the best available cell lines to use in research of this nature.

1.7 **Collagen**

Collagen is the most abundant protein found in mammals, it is synthesised intracellularly where it self assembles. There are multiple types of collagen, all sharing a common triple-helical domain but varying in other structural elements (Kuhn (1987)). These collagen subunits bind at the homogenous domain to create a collagen fibre which possesses high tensile strength and stability, the interlacing of these fibres providing a 3D supportive matrix around cells (Chattopadhyay (2012)). The collagen fibre scaffold renders the ECM that in turn coordinates integration of other molecules and receptors (Aszódi et al (2006)) therefore controlling the rigidity and composition of the cellular environment which has been shown to regulate cellular function and development (Cen et al (2008)). Collagen peptides can form homo- or hetero- trimers, with every variation having different values of tensile strength and rigidity. The concentration and location of each collagen type can impact on the stiffness and stability of the cells and tissues in the body, for example type I and III are the most predominantly found collagens in liver capsules (Van der rest (1991)). Collagen concentrations can vary depending on environment and cellular activity, for example hepatic stellate (HS) cells regulate the polymerization and degradation of collagen fibres in liver cell ECM in response to toxicity and damage. HS cells are able to produce enzymes that digest the collagen matrix, MMPs, which can be inhibited by TIMPs, through this process of degradation, secretion and inhibition all aspects of collagen matrices can be created and remodelled by cells (Friedman et al (2003)). The topology, density and concentration of interlacing collagen fibres, as well as the component types making up the fibres, all regulate the rigidity, stability and mechanical strength of collagen matrices (Franke et al (2013)). The cellular production of collagen and related regulatory enzymes control the concentration in the surrounding ECM, therefore creating and managing the

rigidity of a cells' own environment.

Due to the abundance of collagen in the body it is often used in cell culture to simulate the environment in-vivo. Collagen polymers are able to regulate and maintain cellular differentiation, and because of this collagen has been utilised as a growth scaffold in tissue engineering research (Cen et al (2008)). Cells cultured on collagen layers have been shown to remain viable for longer and retain more in-vivo like function and specialization in comparison to cell grown on polystyrene (Guillouzo (1998), Hewitt et al (2007)). Collagen gels have been observed to promote intercellular interactions in cell culture (Nahmias et al (2007)), this has been linked to the possibility that they experience similar mechanical stability as found in-vivo (Wolf et al (2008)). Sandwiched cells have also been proven to retain polarity to a higher level than monolayer cells, this was conducted using hepatocytes and indicated by the formation of bile canaliculi (LeCluyse et al (1994)).

In-vivo cells are fully encapsulated by collagen, in the ECM, therefore as a further methodology of simulating a more homogenous in-situ environment cells can placed between layers of collagen gels. Hepatocytes have been shown to grow optimally, have sustained viability and retained function, on softer type 1 collagen gels rather than more rigid substrates (Rubin et al (1981)). Collagen concentrations of 0.3% collagen in gels were used for the bottom layer, as is a standard and has been shown to retain hepatoma cells viability (Haramaki (1993)), and a lower concentration of thinner 0.1% collagen gel was used as the top layer to allow for nutrient and oxygen diffusion (Dunn (1989), Foy et al (2004)). This creates a sandwich conformation, as described previously, and surrounds the cell with collagen in a closed system, creating a 3D bioreactor, allowing collagen to interact with the whole cell and more accurately simulating the environment in-vivo.

The liver is an incredibly important organ in the human body; its integral metabolic functions are accomplished mainly by parenchymal hepatocyte cells. The liver is the main site of drug metabolism and nutrient absorption through blood, liver failure occurs when the organ is damaged beyond repair and cannot function. Liver failure causes millions of deaths a year around the world, therefore study of the maintenance of hepatocyte function in-vitro in cultured cells is a crucial subject for drug development, detoxification and tissue engineering. The development and optimization of in-vitro models designed to simulate cells in-situ allows for accurate comparison and experimentation of hepatocyte

cell characteristics in a controlled environment. Cells cultured within a collagen sandwich bioreactor have been proven to retain in-vivo properties for longer than other models. We have decided to utilise this model as it retains the most in-vivo like cellular environment while still allowing for a large scope of research. Using the human HepG2 hepatoma cell line as a model for hepatocytes, we aim to be able to create a model using the collagen sandwich bioreactor which will be able to highlight the relationship between seeding density and cellular viability, morphology, aggregation and cytoskeleton structure.

1.8 **Monitoring cell viability and success of bioreactor**

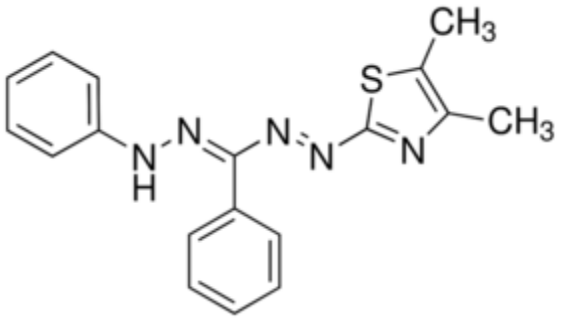
In a sandwich bioreactor model of liver, cells can be observed through the collagen top layer allowing for visual evaluation of cellular adhesion and aggregation however simply observing does not discern functionality or viability. Cell viability can be gauged by two main factors; Intracellular enzymatic function and the presence of an intact plasma membrane. Cells can be tested for both of these viability markers by staining with commercially available compounds, treatment with these chemical dyes can allow for investigation on survival and function of cells. Chemical stains can also be used to visualise specific cellular chemical structures, for example DNA within the nucleus or the actin cytoskeleton within the cytoplasm. In this project several stains were used to ascertain various signs of cell viability and distinguish cell morphology and aggregation.

1.9 **Stains**

MTT

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) has a chemical formula of $C_{18}H_{17}N_{5S}$ and molecular weight of 335.43, it is a membrane permeable chemical that is ingested by cells and stored in their mitochondria and intracellular fluid (Liu et al (1997)). Once within the cell MTT undergoes NADH/NADPH – dependent reduction where the compound is cleaved by reductase enzymes, producing the non-membrane-permeable compound formazan which then builds up in the whole

cell. Formazan is a bright blue colour and insoluble in cells, therefore when the weakly coloured yellow tetrazolium salt in MTT are reduced this discolours the cell. The net positive charge of the whole MTT compound is thought to allow for permeation of the plasma membrane (Berridge et al (2005)). This assay has also been adopted to measure proliferation and toxicity as dye reduction is thought to be proportional to the number of cells that are actively metabolising and proliferating. MTT is a widely available compound generally used to visualise metabolic activity, proliferation and toxicity in a wide range of cells. Although MTT reduction can be attributed to metabolism it only works with specific enzymes and therefore is not a perfect assay for whole metabolism, especially in cells such as hepatocytes that are known to have multiple enzyme metabolism pathways. The method was used in the present study as a rapid simple check that the cells in the cultures were living, prior to initiating more elaborate staining protocols.

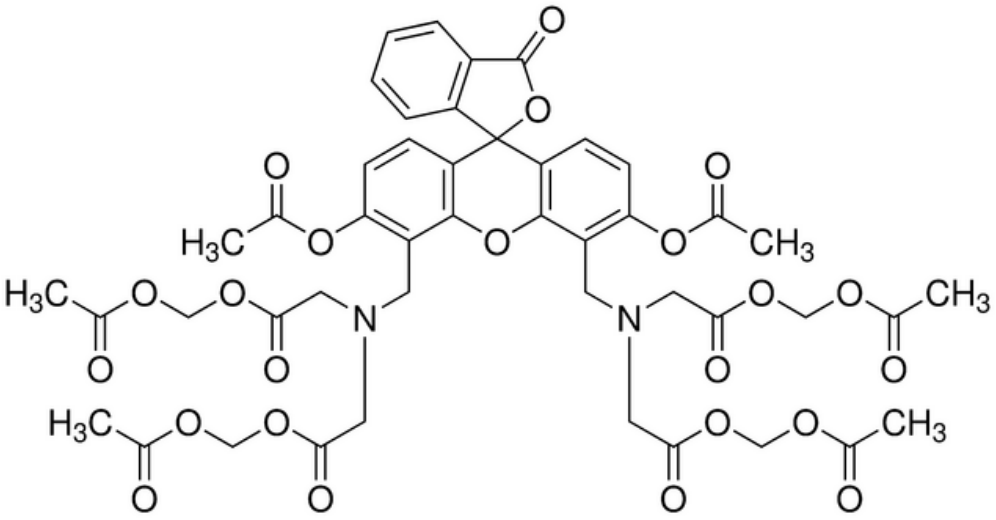


Structural Formula of MTT taken from the Sigma website

Calcein-AM

Calcein O,O′ -diacetate tetrakis(acetoxymethyl) ester (Calcein-AM) has a chemical formula of $C_{46}H_{46}N_2O_{23}$ and a molecular weight of 994.86; it is a cell-permeable dye and is made fluorescent upon acetoxymethyl ester hydrolysis by intracellular esterases. This dye is used to stain for live cells as only metabolising cells with fully intact plasma membranes are able to actively cleave the compound to produce fluorophores and retain them inside the cell. These fluorophores are visualised under certain wavelengths of light, when they are exposed to these specific conditions they re-emit light of a certain wavelength. The wavelength of excitation for Calcein-AM hydrolysis products is 496nm and the wavelength produced is 516nm, a fluorescent green. This

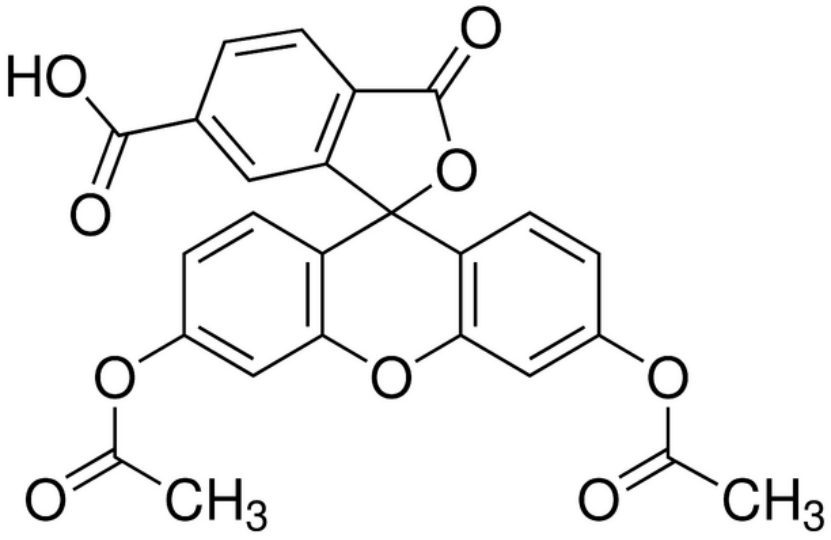
assay was used to detect living cells and as a methodology to observe cell aggregation and distribution.



Structural Formula of Calcein-AM taken from Sigma website

CFDA

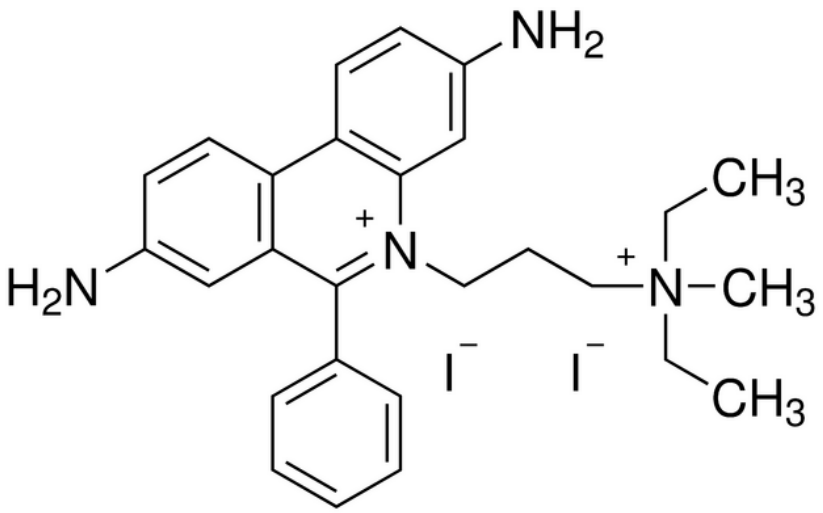
6-Carboxyfluorescein diacetate (CFDA) has a chemical formula of $C_{25}H_{16}O_9$ and a molecular weight of 460.39; it is able to permeate the cellular membrane by diffusion. Once within the cell the CFDA non-fluorescent compound (prefluorochrome) is hydrolysed by intracellular esterases, removing the acetate group and producing more polar fluorophores (Breeuwer et al (1995)). The excitation wavelength of these fluorophores is 492nm and the emission wavelength is 517nm producing a green colour (Interchim handbook). The hydrolysed product of CFDA is retained in the cytoplasm of the cell by the cellular membrane. This staining technique is often used to visualise "live" active cells, as they are deemed viable as they retain fluorophores and have a complete plasma membrane. Fluorescence emissions can be quantified allowing for statistical analysis but in this experiment fluorescent cells were visually (qualitatively) observed for cell morphology, viability, apoptosis and cell adhesion.



Structural Formula of CFDA taken from Sigma website

Propidium Iodide

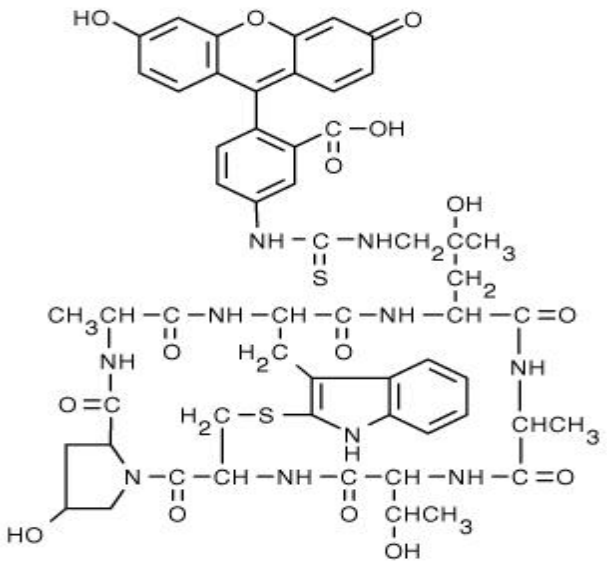
Propidium Iodide (PI) is an intercalating fluorescent agent. It has a chemical formula of $C_{27}H_{34}I_2N_4$ and a molecular weight of 668.4, and is able to insert itself between DNA nucleic acids staining DNA. It is not membrane permeable due to its size, structure and polarity and can therefore only stain DNA, in unviable cells with ruptured cell membranes. The wavelength of excitation for PI is 535nm and the emission is 617nm which is red. This dye was used as a counter stain to show the "dead" cells in culture as it is unable to interact with DNA in viable cells. PI binds to DNA with no sequence specificity, and on binding its fluorescence increases 10-20 times.



Structural Formula of PI taken from Sigma website

Phalloidin-FITC

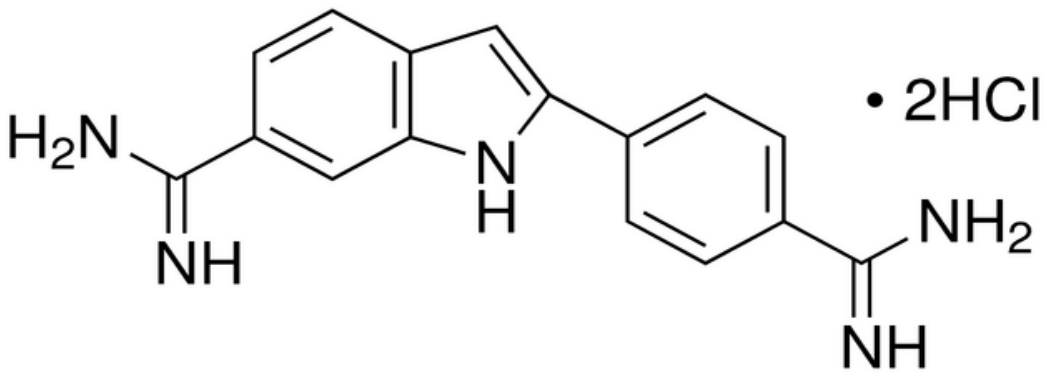
Phalloidin, (Fluorescein Isothiocyanate labelled) (Phalloidin-FITC) is a modified natural dye isolated from *Amanita phalloides*, a mushroom commonly known as the Deathcap; it has a chemical formula of $C_{58}H_{63}N_{10}O_{14}S_4$ and a molecular weight of 1252.44. Naturally Phalloidin is a toxin in the phallotoxin family which binds and inhibits the de-polymerization of F-actin. This functions as a poison and is used as a defence mechanism of the mushroom. Intercalating Phalloidin molecules bind with high affinity between F-actin subunits inhibiting ATP hydrolysis and thus stabilizing actin polymers, therefore stopping movement and paralysing the cell. Phalloidin permeates hepatocytes and hepatoma cells predominantly through the liver specific organic anion transporting polypeptide 1b2 (Thiede and Corwin (2014)). Phalloidin has a molecular formula of $C_{35}H_{48}N_8O_{11}S$ and a molecular weight of 788.9. The FITC compound added to Phalloidin is a derivative of fluorescein, which has a molecular formula of $C_{21}H_{11}NO_5S$ and a molecular weight of 389.38. The combination of the two compounds allows for F-actin polymers to be bound and visualised fluorescently, the excitation wavelength is 495nm and the emission wavelength is 520nm producing a fluorescent green colour.



Structural Formula of Phallodin-FITC taken from Life Technologies website

DAPI

2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) has a molecular formula of $C_{16}H_{15}N_5$ and a molecular weight of 350.25; it is a membrane permeable synthetic stain that has high affinity for A-T nucleotides in DNA. DAPI is able to permeate through viable cell membranes; although it is diffused through fixed cells much more readily and therefore it is optimal to fix cells with formalin prior to staining. Upon binding with DNA this dye fluoresces at an excitation wavelength of 358nm, emitting light at wavelength 461nm which can be seen as blue. This stain is used as a counterstain to Phalloidin and allows for visualisation cell of the nucleus, confirming the positions of cells on the matrices.



Structural Formula of DAPI taken from Sigma website

1.10 Aims and Objectives

This project was designed to investigate, using HepG2 cells as a model for hepatocytes, the effects that seeding density have on cell viability, morphology and shape. To achieve this cells were cultured on 0.3% collagen gels at four different seeding densities, 10, 5, 1 and 0.5 ($x10^4$) cells/cm^2. To optimize experimental similarities between in-vitro conditions and the in-vivo environment, the cells in culture were overlaid with a layer of 0.1% collagen gel producing a 3D sandwich bioreactor. This has been shown to improve retention of cell viability and function of the cells closer to those found in-situ. As a control we will also produce duplicate cell cultures of the same seeding density on monolayers of 0.3% collagen gels. These experiments were run in parallel with another related project aiming to investigate the mechanical properties of collagen gel seeded at identical cell seeding densities. By calculating the compressive modulus of collagen sandwiches with and without cells at various seeding densities this research intended to study the mechanical alterations cells exert on their environment. In this project the aim was to investigate the cellular structure and shape, the actin cytoskeleton, the metabolic activity and viability of cells cultured in a collagen sandwich and observe the differences compared with monolayer cultures. To do this, the HepG2 cells were sandwiched between layers of collagen gel 24 hours after seeding them in monolayer and visualised microscopically. To visualise distribution and aggregation of cells they were treated with MTT, colouring viable cells blue. To determine overall cell structure the cell cultures were stained with FITC-Phalloidin (binds to actin), Calcein-AM and CFDA (a live dead stain) and fluorescent microscopy performed. The actin skeleton structure of the hepatocytes was examined by comparing the structural conformity and rigidity in-vitro with the situation in the liver in-vivo.

2.0 Materials and Methods

2.1 Preparation of HepG2 Human Hepatoma Cell Cultures

1X Dulbecco's modified Eagle's media (DMEM) was bought from Cambrex, New Jersery, USA. The stock 1L bottle was supplemented with 50ml sterile 10% Foetal Calf Serum, 5ml PEST (100 units/ml penicillin and 0.1 mg/ml streptomycin) and 5ml of 1% non-essential amino acids. HepG2 cells were retrieved from cryopreservation and obtained as described in Grant et al (1988). HepG2 cultures were maintained in ~5ml aliquots of the supplemented DMEM. Cultures were incubated at 37 degrees in a 5% carbon dioxide/air humidified incubator and passage between adherent flasks every 3-4 days using versene (EDTA) and 1% trypsin. Cultures to be used in experimentation were split by a ratio of 1:2 the day before experimentation.

Cells were prepared and plated on standard 15mm diameter petri dishes, previous studies and co-workers in the laboratory suggested a cell density of hepatomas in sandwiched bioreactors to be $5x10^4$ cells/cm^2 (Farkas (1998)). To establish whether seeding density on gels impacted on the cell viability, interaction with collagen or intercellular interactions subsequent plates were seeded with densities of 10, 5, 1 and 0.5 ($x10^4$) cells/cm^2 for each experiment. The seeded plates were covered in medium (to ensure the total volume was 2ml) and left for 24 hours in an incubator.

2.2 Collagen Gel Preparation

Collagen type 1 was isolated and prepared from rat tail tendons as described by Elsdale and Bard (1972). Standard 15mm petri dishes were coated with 1ml of a mixture of Collagen, DMEM, 0.4M Sodium Hydroxide and 1:1000 v/v acetic acid, this mixture was brought to pH (~8-8.5) by introduction of droplets of 1M NaOH (Griffiths et al (2008)). The exact concentration of each ingredient was dependent on the required collagen concentration in the gel and the stock collagen concentration, except the volume of DMEM:NaOH which was always 1ml per 10ml of solution.

Example calculation:
Stock solution of collagen gel: 5.88mg/ml
Required concentration of gel: 0.3%
To create a 10 ml mixture for plating on ~ 9 x 9.6cm^2 dishes requires 5.1ml of collagen, 1ml of DMEM:NaOH at a ratio of 1:10ml was then added and the volume made up to 10ml by adding 3.9ml 1/1000 acetic acid.

The reagents were kept on ice, as the collagen would otherwise gel too rapidly upon reaching pH 8 and maintaining a low temperature was observed to inhibit the gelling reaction and allow for timely plating. Alteration of pH was observed by the colour change of phenol red in the DMEM from yellow to pink with the addition of 1M NAOH droplets. The petri dishes were covered with 1ml of collagen gel solution; they were then gently swirled to ensure uniform density of gel layer. The gels were allowed to set in the laminar flow hood for at least 2 hours then treated with 2ml supplemented DMEM solution and left in the incubator for 30 minutes prior to seeding. Post seeding; cells were left to attach in the incubator for 24 hours. The seeded collagen gels were drained of medium and a layer of 1ml 0.1% collagen gel, prepared as described earlier was overlaid on the plate. This layer was then left to gel for 2 hours and then covered in 2ml of medium. The cultures were then left for 24 hours before staining. The top layer was very fragile; therefore gentle pipetting, usually on the edges of plates, was used to avoid rupturing the collagen top layer. All solutions, except the collagen used in the production of these gels was sterilised through

0.2µm filters; the collagen was sterilised before use by centrifugation and was stored at 4°C.

Diagram 1. Methodology of Constructing a Collagen Sandwich Bioreactor

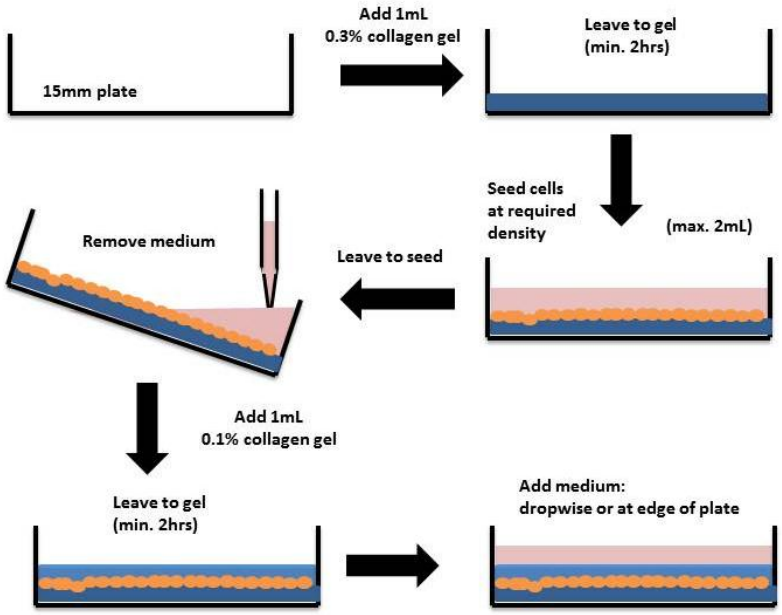


Diagram 1. Steps to seeding cells in a collagen sandwich bioreactor. Firstly add and gel bottom collagen layer, seed with cells, remove medium, overlay cells with top layer, allow to gel and add medium.

2.3 Staining

Reduction assays

MTT solution was made from 0.4143g of stock MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) dissolved in 100 ml of 6.75 pH phosphate buffered saline (PBS) solution and sterilised through a 0.2µm filter. Samples were drained of medium and 1 ml MTT was directly pipetted onto the plates and left to stain for 4 hours in a 37°C incubator. This stain was used to assess the cell viability of the sandwiches. This assay showed that cells were viable, respiring and had acceptable nutrient availability.
Images were obtained using a Moticam 10 (resolution: 10MP, Sensor: cmos) attached to a Motic AE31 confocal microscope, with 10x objective equipped.

Live/Dead Assays

Calcein acetoxymethyl (AM) and Carboxyfluorescein diacetate (CFDA) staining were utilised to establish and visualise the viability of living states of cells. CFDA stock solutions were diluted to 1:100 DMSO to give 25µM solutions, Calcein-AM was diluted to a 1:200 DMSO dilution to give a 5µM concentration. Propidium iodide (PI) staining was utilised as a counterstain to visualize non-viable (dead) cells as it binds to intracellular DNA, and gains entry to the cell through damaged membranes. Hepatoma cultures were first drained of medium and then washed twice with 7.4 pH PBS buffer. As stated previously all solution extraction, introduction and washes were carried out gently using Gilson pipettes to avoid damaging the delicate top 0.1% collagen top layer. The cultures were then covered with 1ml of PI (20µg/ml in 7.4 pH PBS) and left in the dark for 1 minute. The PI was removed and the sandwiches washed with PBS,7.4 pH for Calcein-AM or 6.75 pH for CFDA. 1mL of either of these dyes solutions was then pipetted onto the gels and the cells left to incubate in the dark for 5 minutes. The dye was then removed and the cultures washed with PBS three times, 2ml of PBS was then placed on the sandwiches and the samples visualised using a Zeiss AxioImager ZI, Cambridge, UK fluorescent microscope with a 20x water lens. From this assay we are able to establish cells which are capable of de-esterifying CFDA, and have intact membranes to retain the CF and Calcein and therefore are viable.

Phalloidin-FITC/DAPI Staining

Phalloidin-FITC staining was utilised to visualise the actin structure of the cells in culture. 4',6-diamidino-2-phenylindole (DAPI) was used as a counterstain to illuminate the nuclei of cells in culture. Cell cultures were washed with warm 7.4 pH PBS and then fixed with 1mL 4% formalin in PBS for 20 minutes, in a fume hood. The samples were then washed 3 times with PBS and stained with 0.5ml 1µg/mL phalloidin-FITC and left to incubate for 1 hour in a dark moist chamber. The plates were then rewashed two more times and stained with 0.5ml of 1µg/mL DAPI dilution for 30 seconds. The cultures were washed and visualised using a Zeiss AxioImager ZI microscope with a 20x water lens (20x/0,50 W PH2 from Achroplan) attachment. This assay allowed for the visualisation of the actin structure and nuclei of the cells in a sandwich bioreactor.

3.0 Results

3.1 Initial Findings

From previous research on seeding densities of hepatocytes (Farkas (1998)) and practical work carried out in the lab by co-workers utilising the HepG2 cell line, it was established that 50,000 cells per centimetre squared was an acceptable initial seeding density. To investigate whether seeding density impacted on viability two sets of plates were seeded with 5 x 10^4 and 2.5 x 10^4 cells/cm^2.

Viability was measured qualitatively in these initial studies by observing the distribution of the blue formazan crystals after the reduction of MTT by viable cells. At both seeding densities, as shown on Figure 1, cells were observed to retain high viability 48 hours post seeding, as demonstrated by MTT assay. These two sets of samples were observed to vary in cellular distribution and aggregation; the 5 x 10^4 cells/cm^2samples were seen to experience increased intercellular adhesion, but no apparent difference in relative viability was detectable.

Figure 1. MTT Assay 48 hours Post-seeding, 2.5 x 10^4 and 5 x 10^4 Cell/cm^2 Density Plates

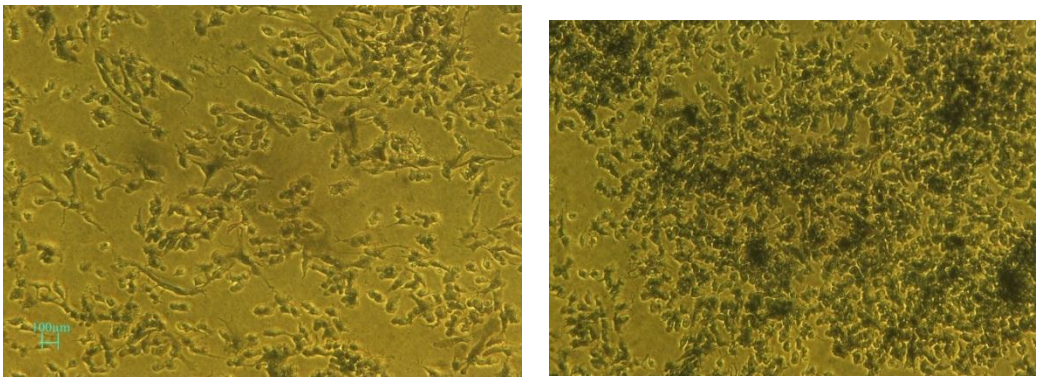


Figure 1. 2.5 x 10^4 cells/cm^2 (left) and 5 x 10^4 cells/cm^2 (right) HepG2 cells, stained with MTT after 48 hours culture on a 0.3% collagen monolayer. Triplicates of each plate were cultured, stained and at least three pictures of each plate were taken. The image shown is a typical representation of cells . This figure shows the viability and distribution of cells. Visualised using Moticam 10 and 10x objective on a Motic AE31. Scale; 3mm:100μm

A 0.1% collagen overlay was added 24h after plating the cells into cultures at both of these cells densities to create collagen sandwich bioreactors which were then visualised under microscopy. Cells sandwiched at a 2.5 and 5 x10^4 cells/cm^2 were visually observed to have a low distribution (Figure 2), aggregating in bulk to specific areas. Cells at both of these densities formed ill-defined clumps making individual cellular observations inaccurate so in further experiments lower cell seeding densities were used.

Figure 2. 24 Hour 2.5 x 10^4 Cells/cm^2 and 5 x 10^4 Cells/cm^2 Cells Collagen Sandwiched Plates

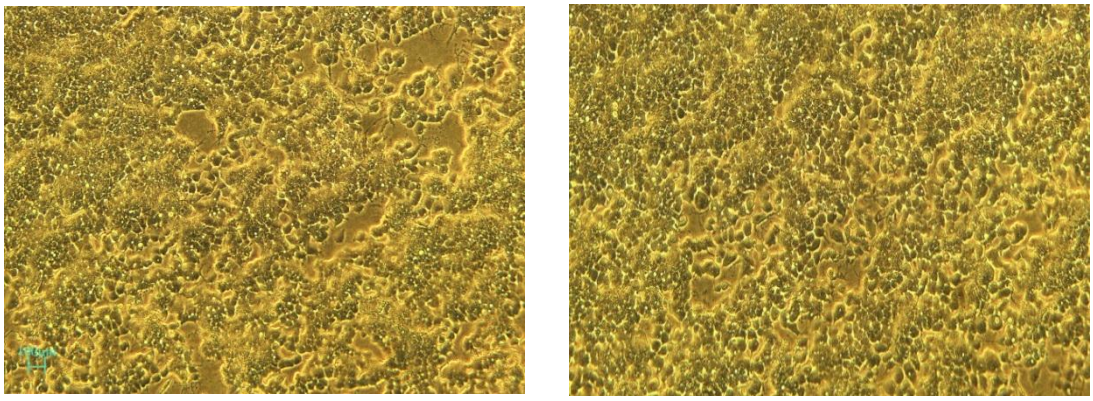


Figure 2. 2.5 x 10^4 cells/cm^2 (left) and 5 x 10^4 cells/cm^2 (right) HepG2 cells, sandwiched for 24 hours with 0.1% collagen after 48 hours culture on a 0.3% collagen monolayer. This figure shows the distribution of cells. Triplicates of each plate were cultured and at least three pictures of each plate were taken. The image shown is a typical representation of cells Visualised using Moticam 10 and 10x objective on a Motic AE31. Scale; 3mm:100μm

From further reading (Du et al (2008), Timpe et al (2008)) and discussion with co-workers in the laboratory it was decided to observe the viability, aggregation and other intercellular interactions at double the recommended density (10 x 10^4 cells/cm^2) and at lower concentrations of cells (1 x 10^4 and 0.5 x 10^4 cells/cm^2). This range of samples was observed to vary in distribution for both monolayer and sandwiched conformation and would therefore yield better comparisons between seeding densities, see Figure 3.
These cell seeding densities will be referred to as 10, 5, 1 and 0.5 (x10^4) cells/cm^2 from here on. For each of the following experiments all samples were made in triplicate and multiple images were taken of each gel, the images shown are typical examples of each sample density.

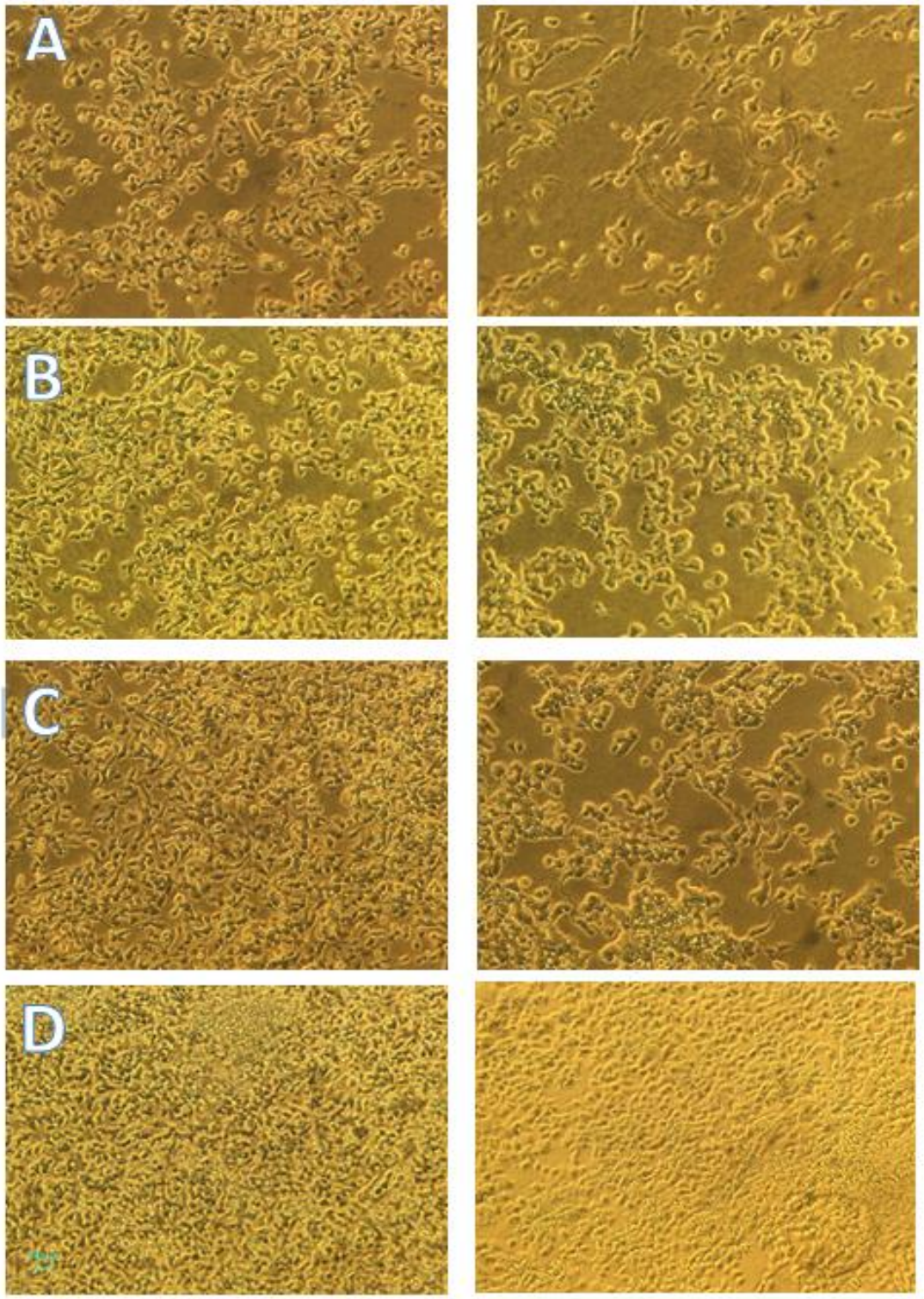


Figure 3. HepG2 collagen monolayer control (left) and sandwiched cell cultures (right) of 10, 5, 1 and 0.5 (x10^4) cells/cm^2 (A-D). Cells cultured on monolayer for 24 hours and then overlayed with 0.1 collagen sandwich layer (renew medium for controls). Visualised using Moticam 10 and 10x objective on a Motic AE31. Scale; 3mm:100μm

3.2 MTT Staining

To evaluate viability and distribution of the cells in collagen sandwiches and monolayer controls, the medium was removed from cultures and MTT was introduced to the plates and left to incubate in the cells. The reduction of the MTT to form the blue salt, Formazan, demonstrated the viability of the cells and their ability to perform intracellular reduction (Berridge et al (2005)). High concentrations of Formazan correlate to large viable cell numbers.

These MTT assays showed that all four seeding densities in this collagen sandwich conformation were viable and received sufficient nutrients to metabolise, as they retained similar Formazan formation as the non-sandwiched controls (Figure 4 and 5).

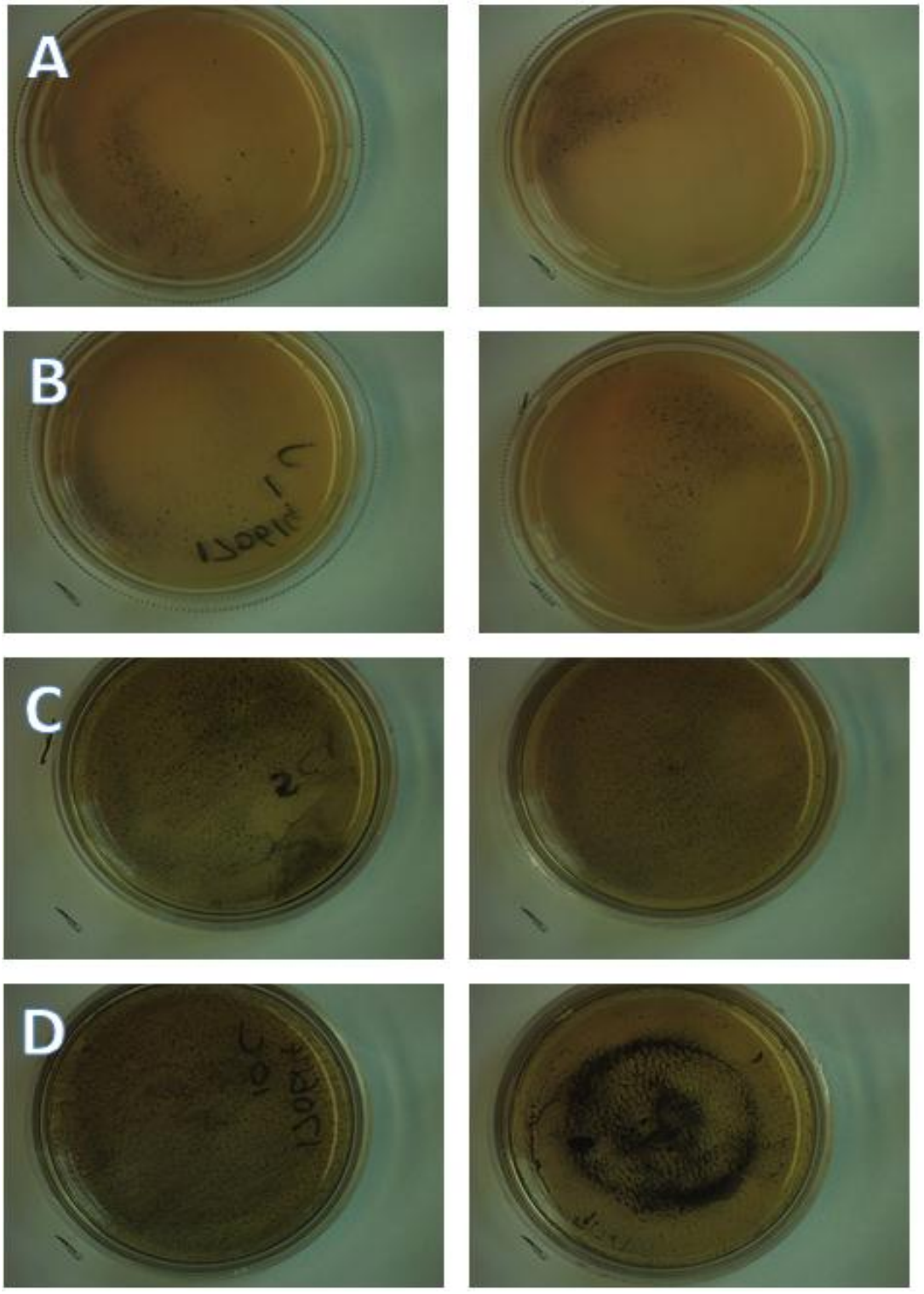


Figure 4. Whole plate images of HepG2 collagen monolayer control (left) and sandwiched cell cultures (right) of 10, 5, 1 and 0.5 ($x10^4$) cells/cm^2 (A-D) stained with MTT. Cells were stained for 4 hours with MTT 48 hours post seeding. Visualised using Moticam 10 and 10x objective on a Motic AE31.

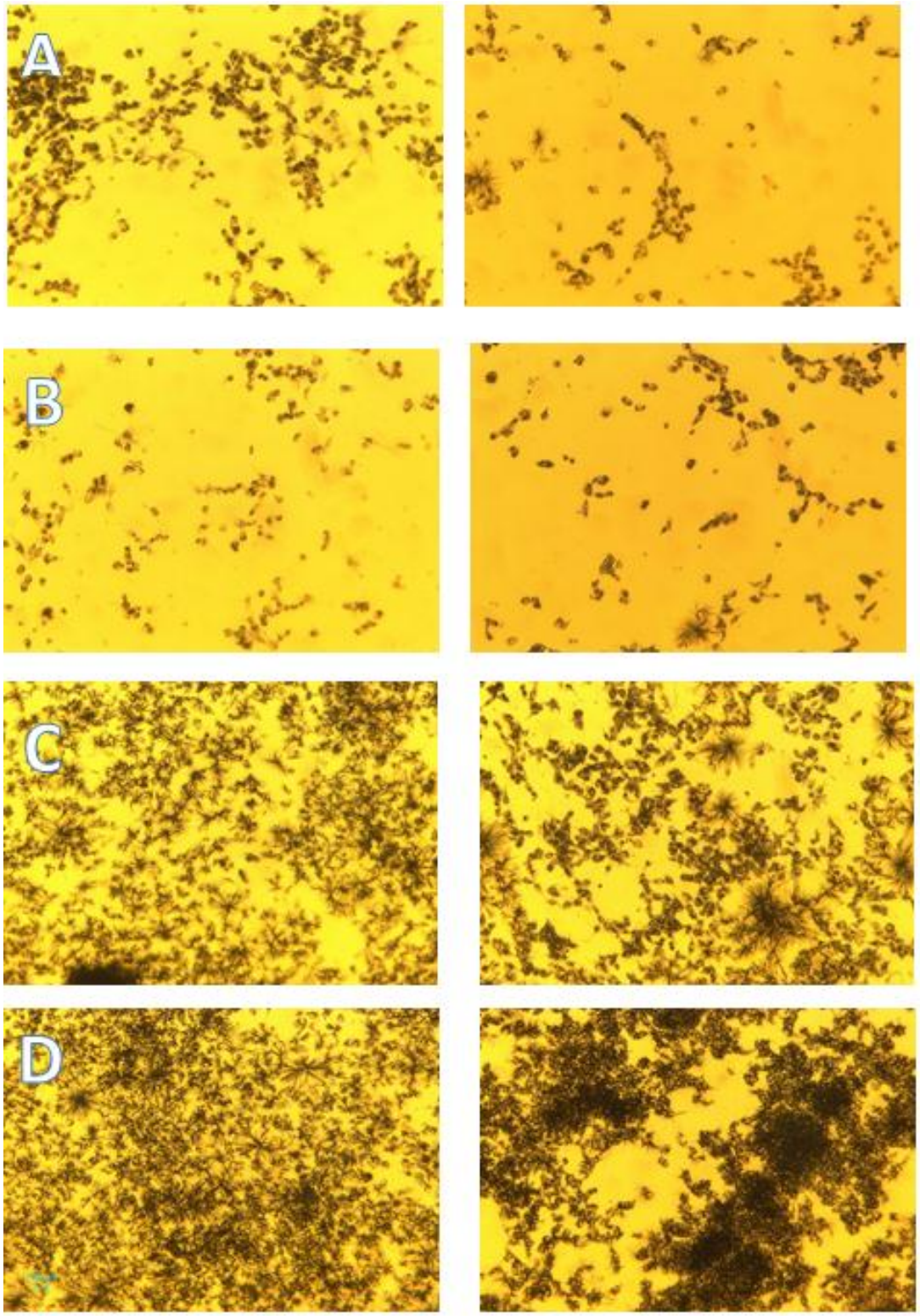


Figure 5. HepG2 collagen monolayer control (left) and sandwiched cell cultures (right) of 10, 5, 1 and 0.5 ($x10^4$) cells/cm^2 (A-D) stained with MTT. Cells stained for 4 hours with MTT 48 hours post seeding. Visualised using Moticam 10 and 10x objective on a Motic AE31. Scale: 3mm:100μm

Generally whole plate distribution of cells at specific densities were similar between controls and sandwiched samples (Figure 4 A-C), except sandwiched cells seeded at $10x10^4$ which aggregated towards the centre of the plates, usually forming a ring (see Figure 4 D). From magnified images intercellular aggregation can be observed on the higher concentration plates, 10 and 5 $x10^4$ cells/cm^2, suggesting higher instances of intercellular interactions, this was seen on both the sandwiched and monolayer plates (Figure 5 C and D).

In the images crystals can be seen alongside cells, most notably in figure 5 C, this could be due to excess MTT stain remaining on the plates or impurities in the dye sample.

3.3 **Calcein-AM and Propidium Iodide staining**

To establish survival states of cells in culture, and also show the presence and activity of functioning esterase enzymes, the samples were stained with Calcein-AM and Propidium Iodide. Calcein-AM dye fluorescently marked viable hepatoma cells green, through the aggregation of hydrolysis product in the cells cytoplasm (Decherchi et al (1997)), while PI molecules bound to DNA fragments in damaged or dead cells were stained fluorescent red. This assay allowed for comparison of the presence of live/dead cells while also allowing for some examination of the morphology of live cells. In parallel to this experiment samples were also dual stained with Calcein-AM, Propidium Iodide and MTT, this resulted in inadequate images, where all that could be seen was a fluorescent green blur.

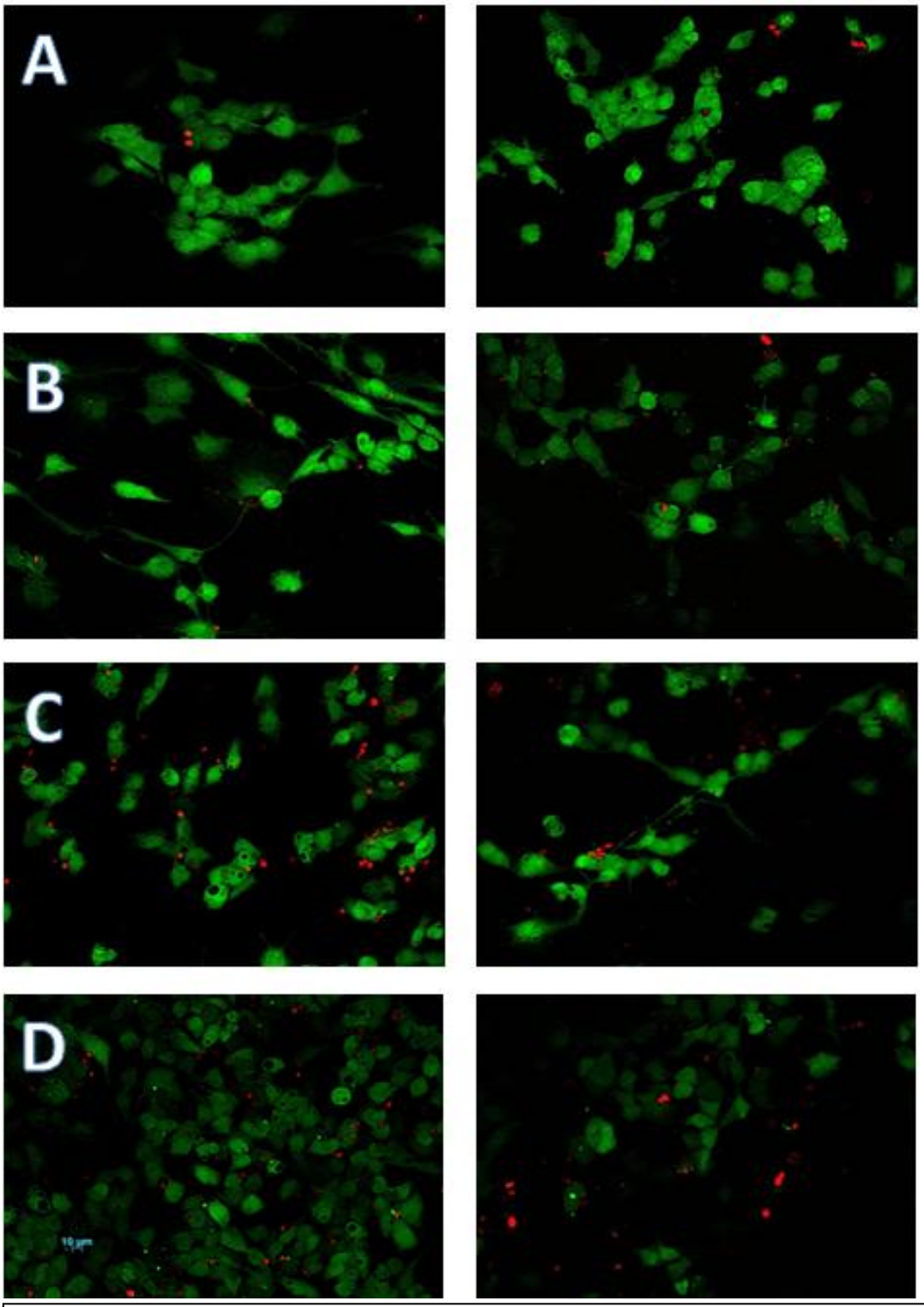


Figure 6. HepG2 collagen monolayer control (left) and sandwiched cell cultures (right) of 10, 5, 1 and 0.5 (x104) cells/cm2 (A-D) stained with Calcein-AM and PI. Cells seeded for 24 hours on a monolayer and then overlaid with 1ml of 0.1% collagen gel, left for 24 hours and then stained. Cells were stained for 5 minutes with Calcein-AM and 30 seconds with PI. Visualised by fluorescence microscopy utilising Zeiss with a 20x water lens attachment. Scale; 3mm:100μm

From the images taken in this staining experiment; sandwiched hepatoma cells have similar morphologies to the monolayer control cells. At 0.5 and 5 x10^4 cells/cm^2 cell density there was very little differences in the live/dead ratio between sandwiched and control samples or among the seeding densities, although the survival was seen to double at 1 x10^4 cells/cm^2 and half at 10 x10^4 cells/cm^2 (Figure 6 A-D also see Table 1). In images taken of cells of 5 x10^4 cells/cm^2

density in collagen sandwiches it was observed that cells produced extensions interconnecting each other (Figure 6C sandwich). On plates of the higher cell concentrations, at 10 x10^4 cells/cm^2 (Figure 6 D), sandwiched hepatoma cells were observed to fluoresce Calcein fluorophores at a (visually) comparatively lower intensity than controls; suggesting less viable cells, lower enzymatic activity, deterioration of the dye, insufficient dye per cell or an interaction of the top collagen layer with the fluorescent dye. Focusing errors and fading of fluorescence did occur in some plates across every seeding density due to the collagen top layer.

Table 1. Estimated Cell Count Of Calcein-AM Sandwich and Control Plates

Cell Density (x10^4 cells/cm^2)	0.5	0.5	1	1	5	5	10	10
Culture Type	Mono-layer	Sand-wich	Mono-layer	Sandwich	Mono-layer	Sandwich	Mono-layer	Sand-wich
Live	38	124	77	127	193	147	563	116
Dead	8	27	26	23	54	37	119	67
Number of Images Counted	3	6	3	4	3	4	3	4
Live-Dead ratio	4.75	4.59	2.96	5.52	3.57	3.97	4.7	1.73

3.4 CFDA and Propidium Iodide

To ascertain the live/dead status of each seeding density and due to the variability of the previous live staining results, the experiment was repeated replacing Calcein-AM with CFDA. CFDA acts to stain live cells through the esterase hydrolysis product, carboxy-fluorescein, being trapped in the cell (Na et al (2012), LeCluyse et al (1994)).

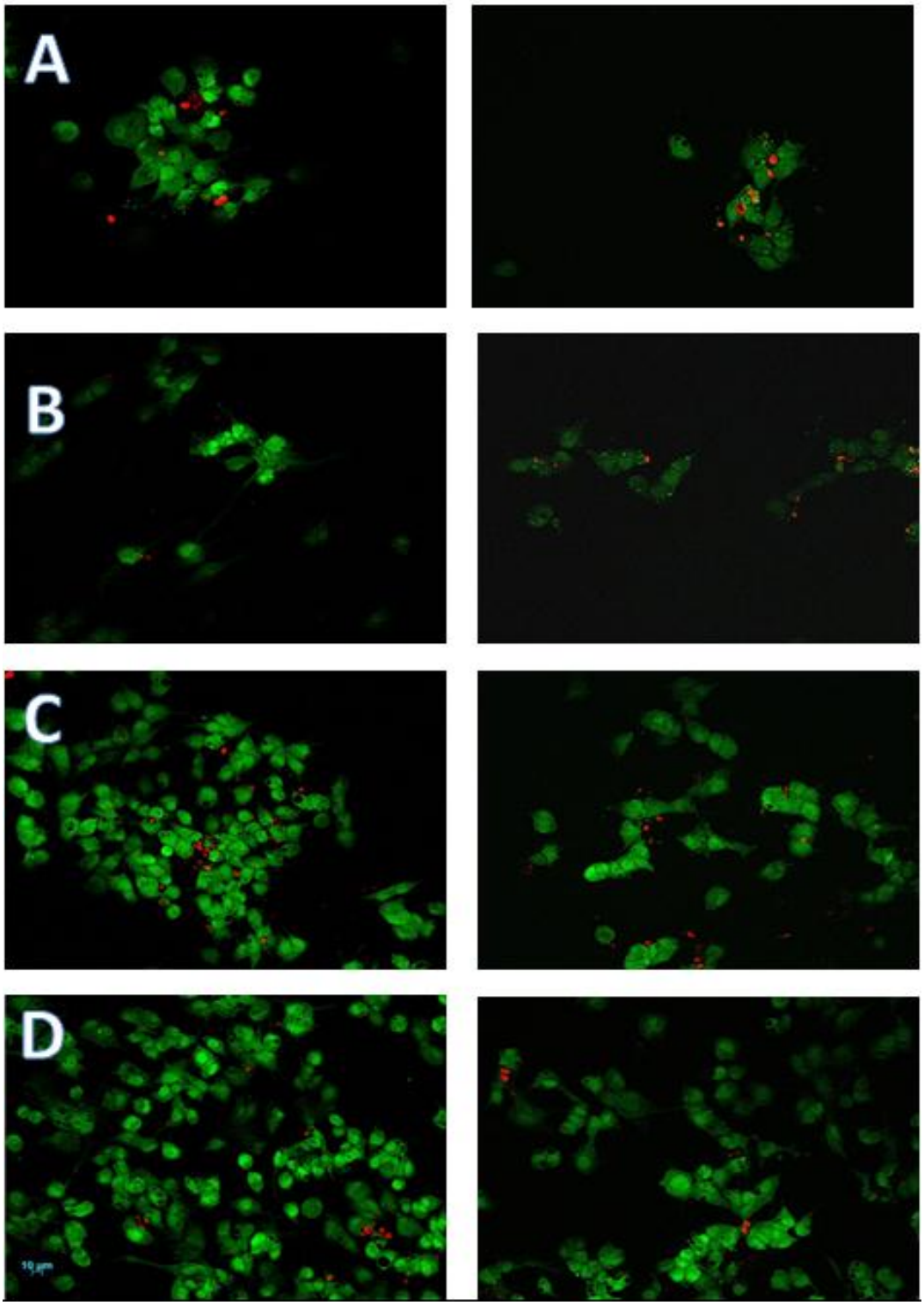


Figure 7. HepG2 collagen monolayer control (left) and sandwiched cell cultures (right) of 10, 5, 1 and 0.5 (x104) cells/cm2 (A-D) stained with CFDA and PI. Cells seeded for 24 hours on a monolayer and then overlaid with 1ml of 0.1% collagen gel, left for 24 hours and then stained. Cells cultures stained for 5 minutes with CFDA and 30 seconds with PI. Visualised by fluorescence microscopy utilising a Zeiss with a 20x water lens . Scale; 3mm:100μm

These low density images show negligible difference in fluorescence and therefore viability between controls and sandwiches of the same cell densities. The fluorescence was observed to be uniform across each seeding density. At low cell concentrations the live to dead ratio between sandwiched and control samples or among the seeding densities was very similar (Figure 7 A-C also see Table 2). At higher concentrations though there were not as many dead cells, which may signify improved sustainability in sandwiched cultures. This could be investigated with more samples, three sets of each sample were created, so a higher sample number would improve validity of this observation. Cells cultured in collagen sandwiches were observed to be slightly more dispersed than controls (Figure 7A) and in high density seeded plates there were visible interactions between cells (Figure7 C and D). In these figures it is possible to observe pseudopodia between cells, these facilitate intercellular interactions as seen most prominently in cells plated at 10 x 10^4 cells/cm^2. The presence of these pseudopodia suggests increased communication between cells at higher seeding densities.

Table 2. Estimated Cell Count Of CFDA Sandwich and Control Plates

Cell Density (x10^4 cells/cm^2)	0.5	0.5	1	1	5	5	10	10
Culture Type	Mono-layer	Sand wich	Mono-layer	Sandwich	Mono-layer	Sandwich	Mono-layer	Sand-wich
Live	29	32	49	96	234	326	320	331
Dead	13	16	13	28	81	104	49	13
Number Images Counted	3	3	3	4	4	4	3	3
Live-Dead ratio	2.23	2	3.77	3.43	2.89	3.13	6.53	25.46

3.5 Phalloidin-FITC and DAPI Staining

To further investigate morphology the seeding cell densities were dyed with Phalloidin-FITC, to stain F-actin with fluorescence green, and samples were counterstained with DAPI, a blue dye which binds DNA in the nucleus of live cells.

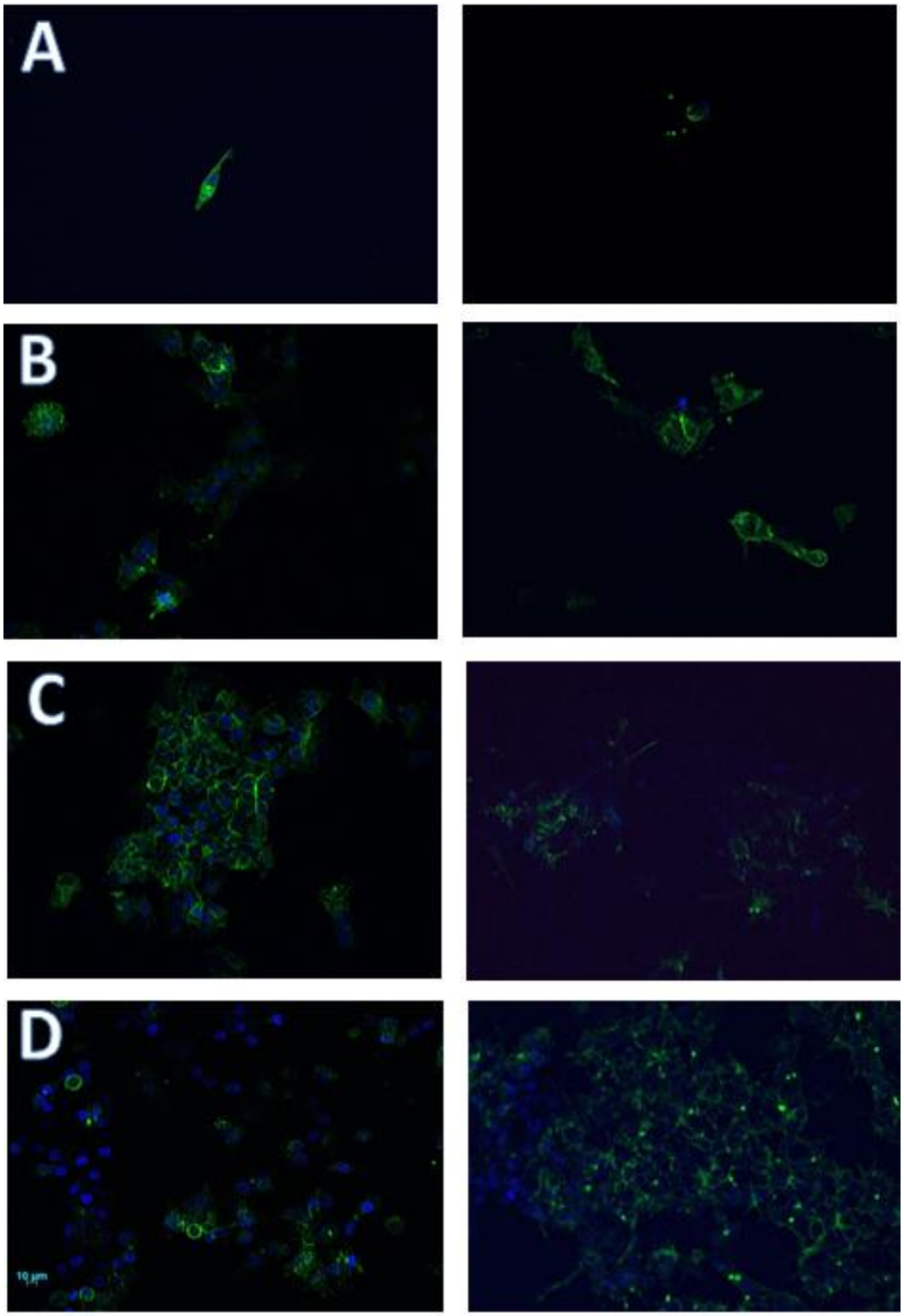


Figure 8. HepG2 collagen monolayer control (left) and sandwiched cell cultures (right) of 10, 5, 1 and 0.5 (x10^4) cells/cm^2 (A-D) stained with PhalloidinFITC and DAPI. Cells seeded for 24 hours on a monolayer and then overlaid with 1ml of 0.1% collagen gel, left for 24 hours, fixed with formalin for an hour and then stained. Cells were stained for 5 minutes with CFDA and 30 seconds with PI. Visualised by fluoromicroscopy utilising a Zeiss with a 20x water lens attachement. Scale; 3mm:100μm

From these images it can be noted that sandwiched cells have a slightly increased spherical morphology (Figure 8A), with larger areas of actin filaments creating pseudopods between cells (Figure 8D). Monolayer hepatoma cell plates were observed to possibly express blebbing which is an indication of apoptosis, this was not observed in the sandwiched hepatoma cells (Figure 8B and D), suggesting lower viability in comparison. Higher density sandwich samples (Figure 8 C and D) showed more DAPI interference causing the blue interference in the background of the images.

3.6 Z-Stacking

The 10 and 5 x10^4 cells/cm^2 density hepatoma plates formed spheroids due to the increased aggregation, 3D formations were visualised through Z-stacking, this programme in the Zeiss microscope enabled imaging at regular distances through the vertical cell cluster.

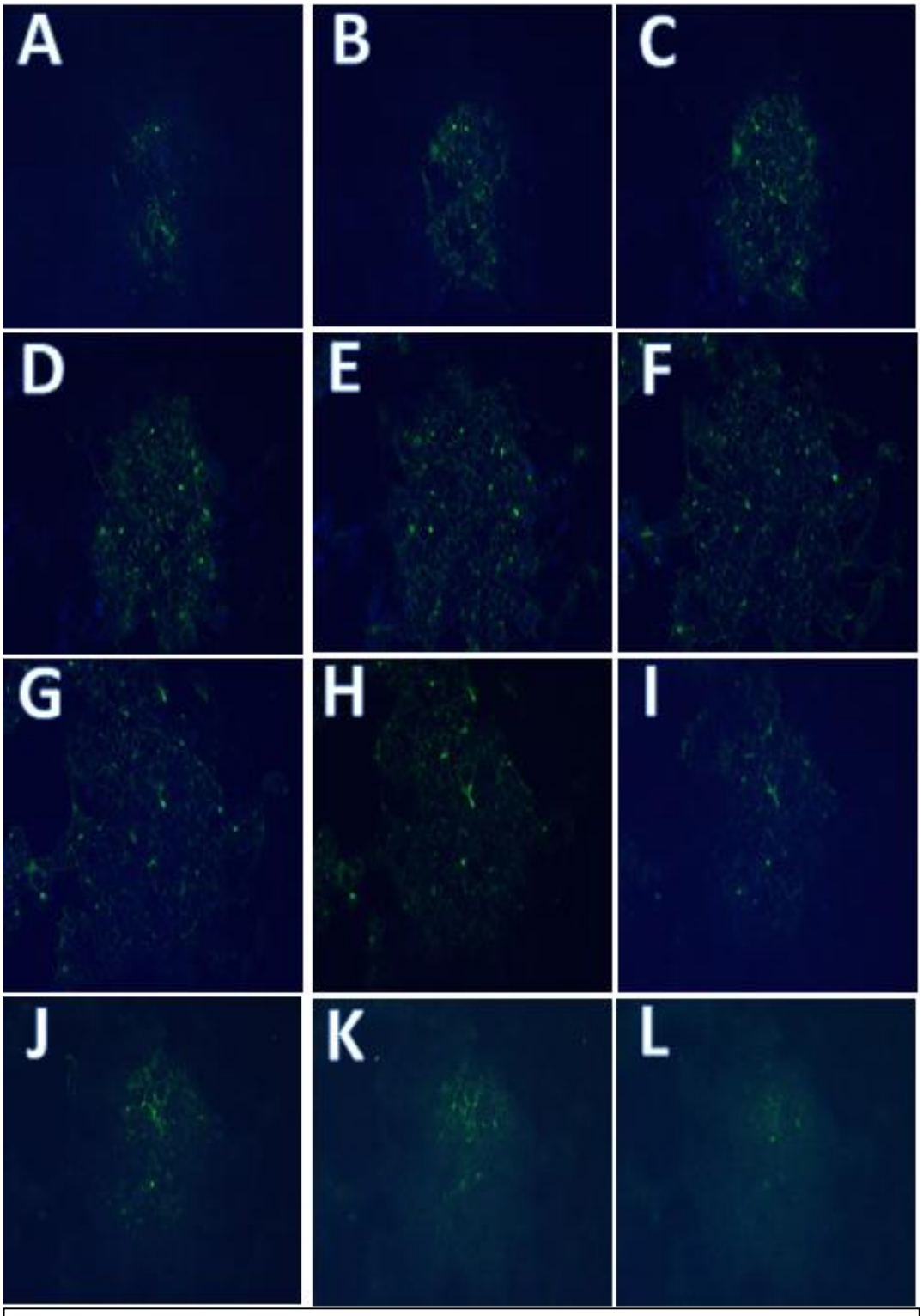


Figure 9. Z-stacked image HepG2 collagen sandwiched cell cultures $5x10^4$ cells/cm^2 (A-L) stained with PhalloidinFITC and DAPI. AL are serial optical sections taken via Z-stacking programme on the Zeiss microscope, each plane shown is a further 5µm deeper into the sample, total depth covered is 55 μm. Cells seeded and stained as before. Visualised by fluoromicroscopy utilising a Zeiss with a 20x water lens attachement. Scale; 3mm:100μm

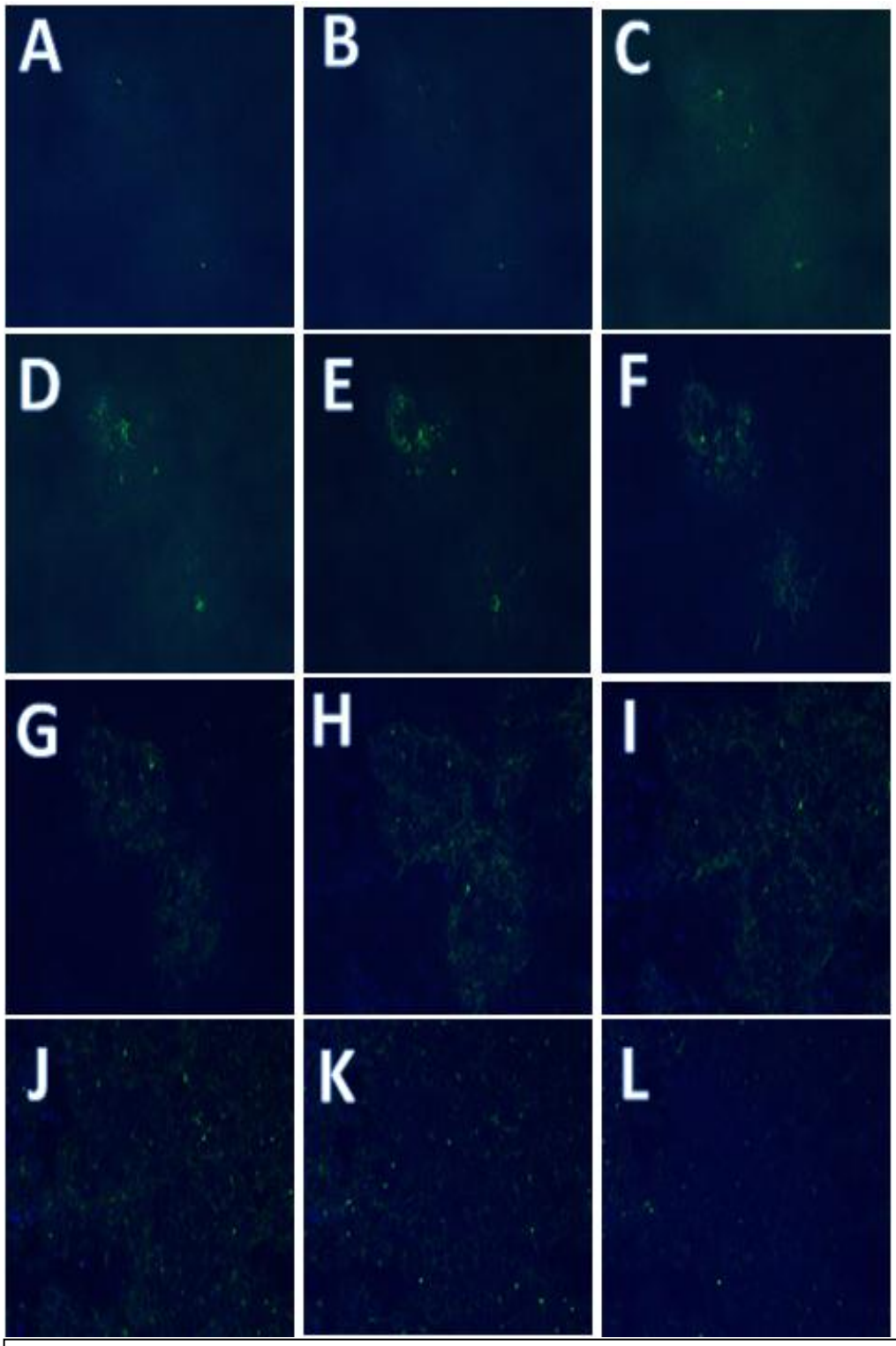


Figure 10. Z-stacked image HepG2 collagen sandwiched cell cultures 10 x10^4 cells/cm^2 (A-L) stained with PhalloidinFITC and DAPI. AL are serial optical sections taken via Z-stacking programme on the Zeiss microscope, each plane shown is a further 5µm deeper into the sample, total depth covered is 55 μm.Cells seeded and stained as before. Visualised by fluoromicroscopy utilising a Zeiss with a 20x water lens attachement. Scale; 3mm:100μm

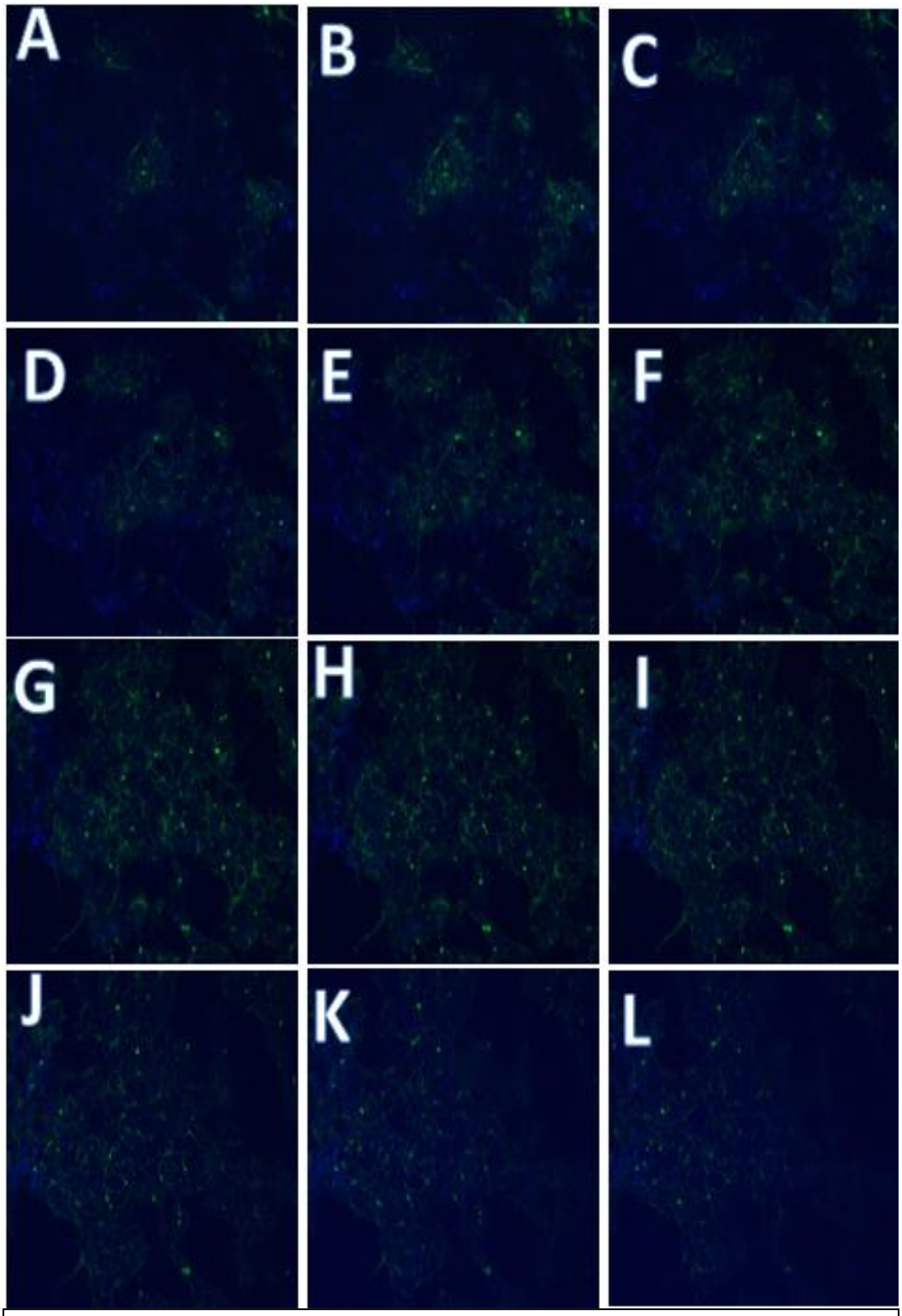


Figure 11. Z-stacked image HepG2 collagen sandwiched cell cultures $10x10^4$ cells/cm^2 (A-L) stained with PhalloidinFITC and DAPI. AL are serial optical sections taken via Z-stacking programme on the Zeiss microscope, each plane shown is a further 1.64µm deeper into the sample, total depth covered is 18.04 *µm*.Cells seeded and stained as before. Visualised by fluoromicroscopy utilising a Zeiss with a 20x water lens attachement. Scale; 3mm:100*µm*

4.0 Discussion

4.1 Summary

The aim of this project was to investigate the effects that seeding density has on cell viability, morphology and shape. HepG2 cells were chosen to model hepatocytes in comparisons between collagen sandwich bioreactors and monolayer conformations. This was done by staining and visually observing the cells, our results show that seeding density and 3D conformation of the surrounding cellular environment may both influence the behaviour and characteristics of human HepG2 Cells. In this research, seeding density had an impact on the aggregation and distribution of cells in culture; plating cells on identical plates at different total cell numbers has shown that HepG2 cells are more likely to form spheroids and participate in increased levels of intercellular communication at higher density populations. The figures presented show that sandwiched cultures show increased aggregation and low distribution while monolayer cells are more even in their dispersal across the whole plate. Cell morphology was observed to be dependent on cellular environment; at lower concentrations individual sandwiched cells were observed to retain the rounded liver phenotype while monolayer cells did not and the latter were observed to bulge and bleb in some plates. These morphologic changes were observed mainly on the Phalloidin-FITC and DAPI staining plates, as shown in images of 1 $x10^4$ cells/cm^2 and 10 $x10^4$ cells/cm^2 (Figure 7 B and D respectively), and have been shown to signify apoptosis and unviability(Saraste and Pulkki (1999)). Sandwiched cultures were observed to form pseudopodia between individual cells, observed in both 5 $x10^4$ cells/cm^2 and 10 $x10^4$ cells/cm^2 density plates on live/dead assays (Figures 6 and 7 images C and D) and in actin stained sandwiches at 10 $x10^4$ cells/cm^2 (Figure 8 D), this was not seen as often but was present on a small number of monolayer plates (Figure 7D). Hepatocytes form lobules in-vivo by aggregating together forming clumps (Michalopoulos and DeFrances (1997)) allowing for increased levels of intercellular interactions between cells. The higher estimated amount of cellular aggregation observed in the sandwiched cells suggests an increased retention in-situ of behaviour patterns in the in-vitro experiments carried out in this study.

HepG2 cells were routinely plated at a density of 5 $x10^4$ cells/cm^2 on 0.3% collagen gels as this was the suggested to be the optimum seeding density for these cells in a 24 hour culture on 15mm diameter petri dishes, as advised by Peter Agbekoh (personal

communication). To examine the relationship between seeding density and cellular behaviour cells were also seeded at 2.5 x10^4 cells/cm^2 on identical petri dishes. Cultures were either sandwiched between collagen layers or stained with MTT as a viability assay and observed to discern distribution or any other differences related to halving the number of cells in culture. These experiments were conducted to establish visually any indicative changes caused by seeding density but did not produce definitive differences in viability or distribution. This could either be due to cellular insensitivity to seeding concentration or that the difference in densities was not large enough to produce any compelling evidence of change. To further investigate the relationship between cell viability and aggregation with seeding density a wider range of cell concentrations were used. Four cell seeding densities, 10, 5, 1, 0.5 (x10^4) cells/cm^2, were chosen to allow for comparative intercellular distribution across the whole plate and aggregation, as observed under microscopy. Comparatively these densities showed larger differences among seeding densities and between the monolayer and sandwiched conformations.

4.2 Cell viability

Sandwich and monolayer cultures were stained with MTT and visualized to compare the viability at different seeding densities of cells in sandwiches to monolayers. This stain also allowed for examination of cellular aggregation as the dye stained the cells blue, allowing the distribution of cells on the plates to be clearly seen. The plates were examined visually to determine viability, quantification of formazan formation could have been carried out but partly due to time constraints it was not. Qualitative examination of the blue staining on the plates enabled us to determine that the cells were viable before embarking on more elaborate staining techniques. Visual analysis of the whole plates showed no obvious difference in viability between seeding densities or between the 2D and 3D models, although in the 10 x10^4 cells/cm^2 sandwich plates it was difficult to discern viability due to cellular aggregation. To further observe intercellular interactions and gauge the extent of distribution, cultures were examined through light microscopy, allowing for more detailed investigation of aggregation and clumping behaviour. Distribution was generally uniform between sandwich and monolayer seeding densities except at the top density, cells seeded at 10 x10^4 cells/cm^2 were observed to aggregate into clusters. These plates were observed to clump more over the whole plate, forming rings around the centre, under magnification

these cells were seen to cluster forming large masses of cells or spheroids.
The reduction of the MTT to the blue formazan salt showed that cells were viable and able to receive sufficient nutrients to allow them to perform NADH/NADHP-dependent metabolism. It should be noted that MTT assay does not measure oxygen transport to the cells or general metabolism. Research has shown that hepatocytes do not actually require high levels of oxygen to remain viable, shown to be tolerant of oxygen uptake rates of as low as 0.38 +/- 0.12 nmol O2/S/10^6 cells (Foy et al (1994)) and liver cells operate multiple metabolism functions and overall metabolism cannot be ascertained from the reduction of a single stain.

4.3 Live/Dead Status of Cells

Calcein-AM

Calcein-AM and Propidium Iodide were utilised to further investigate the viability, aggregation and morphology of cells in cultures. Images of plates with 0.5, 1 and 5 x10^4 cells/cm^2 show no obvious difference in cellular morphology or aggregation between sandwich or monolayer cells. There was variation in the 10 x10^4 cells/cm^2 cell sandwich plates which were observed to have much weaker fluorescence compared to the control; this comparison was conducted by eye but observed consistently over several plates. There is available analytical software, ImageJ software, National Institute of Health, Bethesda, USA (Downloadable at: http://imagej.nih.gov/ij/) as suggested by Professor Helen Grant, but due to lack time it could not be used, and this analysis could be done on the images obtained if required for the continuation of this work by the research group. The difference in emission between monolayer and sandwich cells could be due to any number of reasons; there could be insufficient dye per cell, the collagen upper layer could provide a barrier for the strain to penetrate or a problem with the stain (but this was discounted due to corresponding density monolayer controls which showed no staining problems). Photobleaching was also proposed as a possible reason, it occurs when fluorphores undergo photon induced chemical damage and covalent modification and become unable to fluoresce, which could have occurred due to exposure to light source during image acquisition. The exact parameters of conditions resulting in loss of fluorescence due to photobleaching are unknown (Widengren and Rigler (1996)). Two possible reasons that could be investigated were that the cells could have become dedifferentiated and therefore not metabolised the Calcein-AM or that the high cell number and collagen top layer may

have in some way interacted with the fluorescent dye. A possible method of interaction may have been that the top layer 0.1% collagen gel could form a barrier of diffusion between the cells and the dye; collagen gels have been shown to hinder diffusion more so than some tissues (Ramanujan et al (2002)). The inhibition of diffusion could be due to unassembled collagen producing a physical barrier to molecules. This may have significant connotations in tissue engineering as nutrients and chemical delivery to cells is a major design consideration for all in-vitro models, such as the use of sandwich bioreactors models in researching cellular drug metabolism. The diffusion barrier would cause insufficient cellular exposure to chemicals and could produce incorrect results. Diffusion barriers in-vivo are thought to arise due to unassembled collagen or other matrix molecules not accounted for in current collagen gel in-vitro models, which suggest the need for modification of conformity and molecular composition of gels to improve comparability and therefore validity of future research(Ramanujan et al (2002)).

CFDA

Due to uncertainties on the 10 x10^4 cells/cm^2 Calcein-AM sandwiched plates it was decided to repeat the live dead assay using a different "live" stain, CFDA. The images produced in this experiment were much clearer, showing no differences in fluorescence visually between any seeding density. The live to dead ratios of these cells, shown in Table 2, was also much more consistent, it was observed that only 10 x10^4 cells/cm^2 sandwich cell plates varied in a major way from monolayers as the ratio of live cells doubled. From the images obtained, cells can be observed to project pseudopodia connections with surrounding cells when cultured in the sandwich bioreactor (Figure 7 C and D). Both the 5 x10^4 cells/cm^2 and the 10 x10^4 cells/cm^2 sandwich plates showed higher instances of cell: cell interactions in comparison to their monolayer counterparts (as shown by comparison between left and right images in Figure 7 C and D). The increased intercellular interactions could signify improved cellular communication which suggests better retention of in-vivo phenotype and possibly function. This has been shown in other papers (Moghe et al (1997), LeCluyse et al (2000) and Hamilton et al (2001)) and has been shown to be a marker for improved in-vivo-like cell function expressed in the in-vitro model system.

4.4 Actin Structure of Cells

The actin cytoskeleton was stained and visualised using fluorescence microscopy; this assay was carried out to examine the morphology and possible pseudopodia formation described earlier. By examining the images produced it is also possible to observe that the cells possess intact plasma membranes, confirming that both sandwich and monolayer cells at all densities are viable. In low cell density plates cells were observed to have more of a rounded shape in sandwiches than those cultured on monolayers (Figure 8A), this was not seen in the higher density plates but this may be due to increased intercellular interactions and difficulty clearly seeing individual cell peripheries. The rounded morphology of the sandwich cells resembles that of hepatocytes found in-situ; suggesting that these cells may be able to maintain an in-vivo-like liver cell phenotype more so than the cells on monolayer. Monolayer cells were observed to vary in morphology, with higher concentrations showing cell retractions and loss of the F-actin framework (Figure 8D) .This resulted in larger intercellular spaces, and actin congregating at the cell periphery, unlike in the sandwich cells where networks extend from the centre of the cells. Monolayer cultures showed signs of blebbing, bulges and cell shrinkage which is a morphological marker of apoptosis (Saraste and Pulkki (1999)), while sandwiched cells did not.

Cell contraction and blebbing are late stage indicators of apoptosis, in-vivo these cells would compartmentalise organelles into vesicles to be metabolised by macrophages (Andrade et al (2009)). Cell shrinkage occurs due to intercellular pressure causing rupture of the cytoskeleton, which provides intracellular mechanical support, destabilising the actin cytoskeleton and deforming the cell. Cell blebbing is a consequence of destabilised actin producing intracellular contractile forces on the plasma membrane and creating vesicle bulges. Due to the lack of sufficient ECM on monolayer plates cells could be subjected to higher intercellular forces causing strain on the cytoskeleton and eventual signalling programmed cell death. In-vitro models which do not contain macrophages are unable to metabolise the dead cells and therefore they remain in culture, possibly causing build-up of dead cells lowering oxygen and nutrient availability for other cells or releasing potentially toxic and immunogenic intracellular proteins into the intercellular environment damaging surrounding cells (Fadok et al (1997)).

In high density, 10 x10^4 cells/cm^2, sandwich cell plates, pseudopodia were observed between cells which were not observed in monolayer plates (Figure 8 D). These sandwich

cultures were observed to have larger areas of actin fibres interconnecting in the aggregations of cells; suggesting increased intercellular communication between cells. There was no distinct observable difference of distribution or aggregation at lower concentrations of cells. This suggests that sandwich HepG2 cells at higher densities were more likely to form integrated F-actin networks and were much more interconnected than the monolayer cells, as shown in previous assays.

Overall it was observed that HepG2 cells retained some in-vivo morphology in collagen sandwiches which could possibly relate to intercellular communication and function. In collagen sandwich samples it was observed that intercellular interaction increases in relation to density and morphologically cells retained more of their in-vivo shape. This superior retention of morphology and intercellular communication in sandwich cultures suggests a possible role of collagen 3D culturing in encouraging continued viability of cells, function and morphology.

4.5 Intercellular Communication

As described previously intercellular communication is vital in regulating hepatocytes functionality which is often lost due to dedifferentiation when liver cells are cultured in-vitro, therefore retention of this characteristic is important for in-vitro modelling of liver function. Much of the work in this field has utilised rat hepatocytes, which have been shown to express functional and morphological differences from human hepatocytes, therefore further work with human liver cells is needed. The results of this present study have shown that human cells grown in a 3-dimensional collagen environment are able to retain the ability to form pseudopodia and facilitate possible intercellular communication. To further investigate these findings research could be conducted to investigate the polarity and degree of differentiation in HepG2 cells in culture, possibly investigating the presence of specific specialisation markers such as connecins utilising fluorescently labelled Cnx 32 antibodies, to stain for gap junctions and Z0-1 to mark the tight junctions which form the bile canaliculi, as conducted by LeCluyse et al (1994) with rat hepatocytes. These would show specialised liver cell differentiation, morphology and further suggest retention of liver function. In addition studies of the expression of these proteins could be carried out with immunoblotting for proteins and PCR identification of RNA expression.

4.6 Cell Clumping and the Implication in Bioengineering and Function

Cells have been known to “squeeze” through porous material such as low concentration collagen gels (Even-Ram and Yamada (2005)), like the 0.1% gel utilised in this research. During imaging of collagen sandwich cells at higher densities, it was observed that hepatoma cells clumped and created spheroids that appeared to grow both laterally and vertically. This could be due to cells growing as they would in-vivo to optimise cell-cell surface contact allowing for intercellular communication. Another possible reason for aggregation and vertical cell growth could be that the cells were growing towards the main source of nutrients, the DMEM and oxygen above the top layer of collagen. Either way, hepatic cell arrangements in-vivo are naturally towards aggregation and clumping. Encouraging the cells to behave as they would in-situ suggests that this in-vitro model conformation simulates the in-vivo environment which is advantageous as it allows greater comparison to liver cells in-situ therefore improving the validity of the model for future research.

4.7 Collagen Sandwich Top Layer

Problems arose while imaging cells sandwiched within the two collagen layers, the two main issues were focusing the images and damage or disruption of the 0.1% collagen top layer. Difficulty was encountered while focusing cell layers at specific depth using confocal microscopy, this could be due to refraction of light caused by impurities in the collagen sandwich or the three dimensional configuration of the cells in culture (Artym and Matsumoto (2010)). In higher density plates there were high amounts of non-uniform vertical growth making focusing images harder, to combat this problem Z-stacking was utilised to take multiple images of the gels at set intervals of depth.

The collagen top layer was incredibly delicate, rupturing when small amounts of force were exerted upon it during washes. In order to retain a fully intact top layer all liquids added or removed from gels were done so at the edge of the petri dish very gently by pipetting. This was seen to decrease the sheer stress upon the top layer and minimize tears, and was necessary to retain the closed sandwich system therefore retaining the control of environment and uniform nutrient diffusion to the cells.

4.8 Possible Collagen Interaction With Stains

Dye distortions were observed in multiple plates, the residual dye can be seen illuminating the background of some images. Background dye noise could cause miscalculation in quantitative image based analysis of fluorescence. During the experimentation lower fluorescence was observed in high density Calcein-AM stained cells; this may have been due to chemical degradation of the dyes caused by exposure to light while imaging, although the experiment was repeated using a new Calcien-AM sample from the same stock and showed the same dampening of fluorescence. Dye retention or modification was suspected to be the cause of the failure of the combination attempt at dual MTT-Calcein-AM assay, where no image was acquired due to high levels of fluorescent interference. This may have been due to MTT, Calcein-AM or PI interacting with or not diffusing through the collagen top layer which resulted in a fluorescent image with no discernable features. Although these dyes do not bind to collagen individually they could possibly interact to create an intermediate compound which may modify the collagen.

4.9 Problems and limitations

For the majority of the research the HepG2 cell line was stable producing adequate cell numbers for continued experimentation until the final three weeks of experimentation. From here the cell line was observed to grow at a much slower rate and was passaged once a week to produce high enough cell numbers for seeding. This is unusual in immortalised cell lines and could possibly be due to increased stress caused by passaging. Due to this set back we were unable to repeat the experiments with longer culturing times, this could provide more information on phenotypic retention and maintenance. In further investigations with more time it may be advantageous to alter; collagen concentrations of the gels, culture times or stains used. Due to the limited time, the range of seeding densities was limited to the four values, this could be modified, possibly with the addition of higher seeding densities to further investigate the distribution and intercellular interactions, and to see how large the spheroids/clumps of cells would grow without losing viability. Regulation of spheroid is vital as inappropriately large clumps of cells deprive the inner most cells of oxygen and nutrients resulting in death of the cell (Sutherland (1988)). This results in an accumulation of necrotic cells at the centre of the spheroid which may be

detrimental to cells. By regulating intercellular aggregation it may be possible to maintain an optimum spheroid size and control oxygen and nutrient flow to cells, creating a better spheroid model to investigate cellular phenotypes.

5.0 Conclusion

All tissue engineering aims to improve or treat in-vivo pathologies associated with cellular or tissue problems. To accomplish this models have been developed to simulate the in-situ environment that cells or tissues experience, allowing for accurate comparison between synthetic and natural interactions. In this study we have attempted to investigate sandwich collagen bioreactors as a model of human in-vivo liver cell morphology, viability and aggregation. In summary our results show that human HepG2 cell morphology and intercellular communication is strongly influenced by both the environment and cellular densities of the culture. The findings in this research suggest that lower concentrations (0.5, 1 and 5 $x10^4$ cells/cm^2) of HepG2 cells seeded in collagen sandwiches are able to maintain a rounded in-situ morphology while culturing cells at a high density 10 $x10^4$ cells/cm^2 in a 3D scaffold allows for increased intercellular interactions and aggregation, this in turn may improve retention of in-vivo-like functionality and viability when culturing in-vitro. The increased cell to cell interaction and maintenance of in-vivo morphology could possibly help retain liver function in-vitro for future research aimed at improving the impact and efficiency of studies modelling detoxification of candidate pharmaceuticals and development of synthetic livers tissues.

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